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CHAPTER 1

A Ferment of Fermentations: Reflections on the Production of Commodity Chemicals Using Microorganisms

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Contents

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I. INTRODUCTION

The discovery of penicillin was a landmark event in medicine and microbiology. With its aid, many dangerous infectious diseases became controllable. The use of penicillin by the medical services of the US and UK forces facilitated the recovery of many injured combatants during World War II. For pharmaceutical companies, the success of penicillin stimulated a search for further medically useful materials derived from microorganisms. For example, the discovery of streptomycin opened a new door to the treatment of tuberculosis (Schatz *et al*., 1944). Soon, a Golden Age of Natural Products Drug Discovery was underway as further antibiotics, antiviral and antitumor agents, immunosuppressants, and other materials were obtained in ever increasing numbers from microorganisms.

Penicillin, once a rare drug, difficult to isolate and produce, can now be considered a typical ''commodity chemical''—that is, a commercially important pure chemical compound, that is bought and sold in large amounts in a competitive market. The capacity to produce large amounts of penicillin, beginning six decades ago, was a turning point in the history of the fermentation industry. The commercial production of penicillin was made possible by extensive new developments in the very large-scale growth of microorganisms. These developments have rightly formed the centerpiece of many studies in the history of what has come to be called ''biochemical engineering,'' a term sometimes subsumed into the wider, ''biotechnology.'' Indeed, penicillin has been termed ''A Paradigm for Biotechnology'' (Mateles, 1998).

The drama of the penicillin story tends to overshadow the preceding decades of development in industrial microbiology. In this essay, we look backwards at commodity chemical production by microorganisms before the 1940s. We make no attempt to be comprehensive. Essentially, this is an eclectic essay highlighting features that we feel are of special interest and significance or that have otherwise been overlooked. Our main focus is on organic acids, solvents, and penicillin. The production of amino acids, polysaccharides, vitamins, enzymes, and other commodity chemicals by fermentation is not discussed here, as many reviews on this topic are

available (Bruins *et al*., 2001; Demain, 2000, 2006; El-Mansi *et al*., 2006; Headon and Walsh, 1994; Lynd *et al*., 1999; Macauley *et al*., 2001; Magnuson and Lasure, 2004; Saha, 2003).

The progression from a concept—that penicillin might be a useful therapeutic agent—to its production as a pure, usable drug reliably manufactured on a very large scale, occurred in a period of only a few years, roughly from 1940 to 1947. It was a massive, multidisciplinary undertaking, involving biochemists, biologists, chemists, chemical engineers, clinical microbiologists, and microbiologists, with overall administration and much financial support from pharmaceutical companies and the governments of the United States and United Kingdom. In 1947, 13 USA manufacturers made 510,000 pounds (about 2.3×10^5 kg) of penicillin at a bulk price of \$3,800 per 10^9 Oxford units (\$5.67 per kg). Two decades later, the number of American manufacturers decreased to five, while the annual production increased to 1,749,000 pounds (about 7.9×10^5 kg) and the cost decreased to \$21.75 per 10^9 Oxford units (\$0.03 per kg) (Mateles, 1998). By the beginning of the 21st century, the total annual world market for β-lactam antibiotics (penicillins, cephalosporins) was about \$15 billion (Elander, 2003).

Before the discovery of penicillin, organic chemistry had dominated the pharmaceutical industry. Indeed, many distinguished scientists believed that an economically feasible chemical synthesis of penicillin would replace the use of living microbial cultures. This option was extensively pursued, in secret, both in the United Kingdom and the United States, roughly from 1943 to 1946. However, as noted by the distinguished chemist, R. B. Woodward, in his 1965 Nobel Prize lecture, ''. . . despite the best efforts of probably the largest number of chemists ever concentrated upon a single objective the synthetic problem had not been solved when the program was brought to a close at the end of the War'' (Woodward, 1972).

The search for a chemical synthesis was largely abandoned because it became apparent that not only were microorganisms capable of producing an astonishing array of useful bioactive natural products, but that the traditions of fermentation biology could be refined to meet new standards of reliability and scale. For microbiologists, whose jobs had been concentrated in hospitals and public health laboratories, the era opened lucrative new avenues of employment and required new modes of professional organization. The Society for Industrial Microbiology was founded in 1949 to provide a professional forum for the new breed of microbiologist. Like all economic and scientific revolutions, however, there were many contributing forces that led to the ascendancy of industrial fermentation; it is important to remember how much industrial scale fermentation had been conducted before penicillin was known. Coming back to the present, it is interesting to note a renaissance of natural products as drug candidates, perhaps by use of combinatorial chemistry or biochemistry

(Bentley and Bennett, 1999) and diversity-oriented chemical synthesis (Paterson and Anderson, 2005).

II. WHAT IS FERMENTATION? WHAT IS A FERMENTATION INDUSTRY?

The word, ferment, a substantive and also a verb, is derived ultimately from Latin, *fermentum*, root of *ferv-ēre*, which means ''to boil,'' and was used to describe leaven or yeast that showed a boiling action. The noun form was used in alchemy and acquired other meanings such as agitation and excitement; the verb form was also used in metallurgy and chemistry. Samuel Johnson in his famous Dictionary quotes Boyle's work, which states that fermentation is ''A slow motion of the intestine particles of a mixt body, arising usually from the operation of some active acid matter, which rarifies, exalts, and subtilizes the soft and sulphureous particles; as when leaven or yest rarifies, lightens, and ferments bread or wort.'' (Johnson, 1755, abridged 1843). The Oxford English Dictionary defines fermentation as a process ''of the nature of that resulting from the operation of leaven on dough or on saccharine liquids.''

As the biochemical processes by which yeast produced ethanol and CO_2 from carbohydrates were explored, this activity was referred to as fermentation, presumably an extension of the use of fermentation to describe the manufacture of beer and wines. Similarly, the formation of other materials, such as lactic acid, by microorganisms was also described as fermentation and qualifying adjectives were used—alcoholic fermentation, lactic fermentation, and so on.

The study of fermentation in the 19th century was long and complex, with Louis Pasteur as a major participant. In 1861, he described the transformation of sugar, mannitol, and lactic acid to butyric acid as due to a ''butyric ferment'' that was further described as a motile ''infusorian.'' Remarkably, these infusoria not only lived in the absence of air, they died in its presence (Pasteur, 1861). Pasteur said this was ''the first known example of animal ferments, and also of animals living without free oxygen gas.'' He soon named the infusoria as *Vibrion butyrique* (*sic*) (Pasteur, 1861) but in 1880 this bacterium was renamed as *Clostridium butyricum* by Adam Prazmowski. As further processes not requiring oxygen gas were recognized, Pasteur coined the words ''aérobie'' and ''anaérobie'' to designate life in the presence and absence of oxygen, respectively.

Pasteur is often credited with the aphoristic phrase, ''Fermentation is life without air'' (Vallery-Radot, 1960, p. 220) apparently from his famous publication, ''Études sur la Bière'' (Pasteur, 1876): ''En résumé, la fermentation est un phénomène très-général. C'est la vie sans air, c'est la vie sans gaz oxygène libre. . . .'' In translation, ''fermentation is a very general

phenomenon. It is life without air, it is life without free oxygen gas. . . .'' This lengthy sentence, hardly aphoristic, continues with another 50 words of Pasteurian majesty before coming to an end.

Not everyone accepted Pasteur's view; the scientific debate was long and vituperative. Dubos, one of Pasteur's more eloquent biographers, has this commentary: ''because Pasteur was convinced that fermentation could be more profitably considered as a function of life than as a chemical reaction, and because his opponents refused to meet him on this ground for reasons of scientific philosophy, there arose a battle of words in which many of the most vigorous minds of the nineteenth century took part'' (Dubos, 1950).

The distinguished physiologist, Claude Bernard, became interested in fermentation late in life. Bernard stated that fermentation ''se fait sans fixation d'oxygène'' (proceeds without fixation of oxygen) (d'Arsonval, 1937), and came to believe that alcoholic fermentation did not require a living cell. His evidence for this was not straightforward nor did he publish it. However, after Bernard's death in 1878, various notes, thoughts, and unpublished data were found by d'Arsonval. To Pasteur's distress they were published by M. Berthelot (a rival who doubted Pasteur's conclusions) under the title, ''La Fermentation Alcoolique. Dernières Expériences de Claude Bernard'' (Berthelot, 1878). The final section of this paper referred directly to Pasteur's theory and was titled ''Théorie de la Fermentation Alcoolique.'' He listed five objections and stated that the theory was destroyed. The first objection was as follows: ''Ce n'est pas la vie sans air; car à l'air comme à l'abri de son contact, l'alcool se forme sans levure.'' (It is not life without air, as, in contact with air or not, alcohol is formed without yeast). Needless to say, the evercombative Pasteur vigorously attacked both Berthelot and Bernard in the Académie des Sciences. Given that Bernard was dead, it was a weird polemic, ''in which one of the main protagonists was in the grave and appeared only in the form of a few posthumous notes'' (Dubos, 1950).

Pasteur quickly demonstrated that Bernard's experimental techniques were deficient. Bernard had claimed, for example, that although fermentation occurred in the juice of crushed grapes, he could not find evidence for the presence of yeast. Bernard concluded that yeast was a consequence of, and not the originator, of fermentation. Looking back with hindsight and generosity one can conclude that Bernard had, perhaps, to some extent foreseen that the conversion of sugar to alcohol could, in fact, be accomplished by a collection of ferments (enzymes) even in the absence of living yeast. This concept was finally verified by Buchner's famous discovery of ''zymase'' in 1897 (Cornish-Bowden, 1997).

Pasteur had investigated the manufacture of vinegar; in the process, he identified a microorganism, ''*Mycoderma aceti*,'' as the causative agent and thereby showed that the process was aerobic. In a lecture before the Mayor

and President of the Chamber of Commerce at Orleans, November 11, 1864, he was recorded as follows: ''The Mayor and the President of the Chamber of Commerce having heard that I had studied the fermentation which produces vinegar have asked me to lay before the vinegar makers of this town the results of my work'' (Vallery-Radot, 1960, p. 148). It appears, therefore, that Pasteur's view of fermentation encompassed an aerobic transformation carried out by a microorganism. This wider meaning of fermentation as almost any microbial transformation under either aerobic or anaerobic conditions persisted in Pasteur's lifetime and to the current era, existing side by side with the more narrow, ''microbial transformation of substrates under anaerobic conditions.''

Nowadays in industrial microbiology, the term fermentation is used in a broad way to describe all processes that are carried out in large tanks similar to those used in ethanol fermentations. Writing in his classic text, ''Chemical Activities of Fungi'' in 1949, the distinguished mycologist, Jackson Foster, noted that he used ''fermentation,'' in the colloquial sense, ''meaning the formation of some product by a microbiological process.'' He added, however, that the formation of citric acid by fungi was ''an oxidation, not a fermentation, in the Pasteurian or scientific sense'' (Foster, 1949).

In contemporary technical dictionaries, both meanings are attached to fermentation. One meaning emphasizes the anaerobic breakdown of glucose to lactate or ethanol while the second more broadly encompasses ''the use of microorganisms or cultured cells to produce useful materials, such as antibiotics, beverages, enzymes, and some commodity chemicals'' (Smith *et al*., 2000). In describing the early application of microbes to the production of commodity chemicals, we will use the big-tent definition.

III. WHEN DID THE PRODUCTION OF COMMODITY CHEMICALS BY MICROORGANISMS BEGIN ?

The rapid development of penicillin as a commodity chemical produced by microbial fermentation owed its success to two great traditions in applied microbiology. The older tradition is the application of microorganisms since antiquity for the production and preservation of food and fluids (e.g., bread, cheese, sauerkraut, vinegar, yogurt, beer, cider, kumiss, saké, wine). The pleasures afforded by the various fermented foods and beverages are due not only to the inebrietory potential of the alcoholic beverages but also to the fact that they are complex and savory mixtures of many components. Clearly, a 50% solution of absolute ethanol in distilled water would never substitute for a single malt scotch whisky! Food and beverage fermentations are usually produced by empirical operations. However, two of the ancient technologies forming complex

mixtures have been reworked as industrial operations producing pure products—acetic acid (an extension of vinegar production) and ethanol (an extension of the production of alcoholic beverages). Distillation has been used for centuries to produce ''spirits'' such as brandy and whisky with much higher ethanol content (40–50%) than that of wines (5–18%) and beer (4–8%). Indeed, one name applied to ethanol was ''spirits of wine.''

From the beginning, the second stream of experience focused on the goal of obtaining pure compounds from microbial fermentations. It had originally two major tributaries: one bacterial and the other fungal. (In more recent times, other cultivated cells have also been used).

A. Initial use of bacteria

The bacterial tributary originated in studies on lactic acid (1780) by Carl Wilhelm Scheele (1742–1786). Present in soured whey, lactic acid was called Mjölksyra (''acid of milk'') in Scheele's native Swedish. Some decades later, the ''fermentation'' souring the milk was investigated in some detail; materials such as calf's rennet, dog stomach, bladders, casein, and malt sprouts were active agents for ''fermentation.'' By 1841, French workers, F. Boutron and E. Frémy, had developed a consistent process in which milk, containing additional lactose, was kept in an open vessel at 15–20 °C; as souring and acid production developed, sodium bicarbonate was added (Benninga, 1990). These workers could produce lactic acid on ''a kg scale, even a 100 kg scale'' but the process was long and tedious. Since animal products were not used, it must be assumed that microorganisms from the air were responsible. If only these workers had used a microscope they would have preempted Pasteur's later discovery (explained later). Importantly, the new product, lactic acid, obtained from the ''fermentation'' process began to enter the ''growing market of new drugs and chemicals'' (Benninga, 1990).

In 1854, Pasteur by then distinguished for his work on molecular chirality, was appointed as a Professor and Dean of the Faculty of Sciences at the new University of Lille. Lille was a center for agricultural and food industries, as well as for the production of ethanol by fermentation of beet juice. Pasteur investigated a new problem in the alcohol fermentation that resulted in a spoiled product. He discovered that another fermentation, producing lactic acid, was competing with the yeast, alcoholic fermentation (Pasteur, 1857). Pasteur isolated the ''new yeast'' although he was not actually dealing with a pure culture in today's sense of the term. However, pure cultures of the bacterium, *Bacterium lactis* (now *Streptococcus lactis*), responsible for the souring of milk, were isolated by Lister (1878). They were derived using a dilution method prior to the more famous work of Koch in which bacterial colonies were observed on slices of potato or by streaking on a gelatin plate (Lechevalier and Solotorovsky, 1974).

Meanwhile, across the Atlantic, Charles Ellery Avery obtained US patents in 1880 and 1881 and by 1882 had established a factory for the manufacture of lactic acid by fermentation, in Littleton (near Concord), Massachusetts. The patented product was used to form a baking powder. This, the first example of a large-scale fermentation to produce a pure (or nearly pure) commercial product, was successfully developed in the absence of electricity. The Littleton factory has been lovingly described in detail in the book, ''*A History of Lactic Acid Making*'' (Benninga, 1990). For us, to attempt another detailed description would be superfluous. (An aside.—This book is badly named, since it contains many fascinating stories on other fermentations. John D. Bu'Lock reviewed it in 1991 and in his typical, inimitable fashion, urged that ''anyone interested in (*i*) real biotechnology, (*ii*) real chemical industry, and (*iii*) real adventure stories ... should go out and get a copy'' [Bu'Lock, 1991]. We concur, *A History of Lactic Acid Making* is a classic.).

The Avery factory was only a partial success and it closed around 1889. Some years later, a new lactic acid factory was begun at C. H. Boehringer Sohn in Nieder-Ingelheim, Germany; this company obtained a baking powder patent in 1895 and was able to produce about 4 tons per month using small wooden vats. In the United Kingdom, commercial lactic acid production began in 1894 (Benninga, 1990). The large scale production of lactic acid by fermentation has had, over the years, several ups and downs, but has continued worldwide essentially to the present. One interesting development coming out of the distillery industry was the discovery of a new bacterial species that produced levorotatory lactic acid. This species, named *Bacillus delbruckei*, was isolated from sour mash independently by Lafar and Leichmann (Benninga, 1990). In recent times, the fungus, *Rhizopus oryzae*, also has been used for lactic acid production (Magnuson and Lasure, 2004).

B. Initial use of fungi

Just after Avery had shown that bacteria could be used for industrial purposes, attention turned to fungi. Oxalic acid had long been known to be present in plants and some fleshy fungi. In 1886, DeBary discovered that salts of oxalic acid were also formed by filamentous fungi, apparently by incomplete oxidation of the carbohydrate in the medium. Carl Wehmer (1858–1935), who had majored in botany and chemistry at the University of Göttingen, hoped to use fungi as models for plants, and to this end studied oxalate formation by fungi (Jahn, 1934). Enamored by his model system, he became a mycologist for the rest of his life. In 1890, he spent two semesters with Wilhelm Pfeffer at the Botanical Institute in Leipzig studying oxalate formation. It was in Leipzig that he did initial experiments on the formation of citric acid by fungi (Wehmer, 1893).

Citric acid had been obtained much earlier by Scheele who treated boiling lemon juice with powdered chalk. With considerable foresight, Scheele saw a potential use in the soft-drink industry: ''When one quintin (about 4 g?) of it is ground with six lod (about 15 g) of sugar in a glass mortar, a delicious lemonade powder is obtained, which does not become moist; two spoonfuls of this are dissolved in one quarter of spring water...'' (Benninga, 1990). Indeed, not only is there now a major market for citric acid in beverages, food and candy, but it is also used in pharmaceuticals, in cosmetics, and in other industrial applications (Kapoor *et al*., 1982).

Wehmer isolated two citric acid-forming fungi and created a new genus, *Citromyces*, to house them. *Citromyces pfefferianus* was named to honor Pfeffer and *Citromyces glaber* was named in a more pedestrian fashion, apparently from Latin, *glaber*, smooth. In modern nomenclature, these organisms were most likely to have been *Penicillium frequentans* and *Penicillium spinulosum*. Under favorable conditions, 50% of substrate sucrose was transformed to citric acid (neutralized with calcium carbonate). In one trial, Wehmer obtained 6 kg of citric acid from 11 kg of sucrose; a detailed experimental protocol was provided by Benninga (1990). He realized the practical importance of his work and patented a fermentation process using surface cultures of his organisms in Great Britain (patent number 5,620, 12–9-1893), France (patent number 228,554, 3–11–1893), Germany (patent number 72,957, 2–20–1894), and the United States (patent number 515,033, 2–20–1894).

In a later German patent, the organism used was said to be *Mucor piriformis* (*M. racemosus*) (Challenger, 1929) whereas the 1894 US patent 515,033 describes ''certain fungi (*Champignons*) particularly of two hyphomycetes discovered by myself and called cytromyces (*sic*).'' The fermentation was to be carried out in ''large vessels containing fifty or more liters of sugar solution'' and isolation was best accomplished as the calcium salt.

The first technical production of citric acid was achieved in 1892 at a factory of Fabriques de Produits Chimiques de Thann et de Mulhouse in Thann, Alsace (Wehmer, 1933). This company made tartaric acid and other chemicals for the textile industries. As a result of the Franco-Prussian war, Alsace was at that time part of Germany. The fungi were grown in 1 m^2 trays (''*flachen Gefässe*'') fabricated in lead. If they contained the fifty liters of culture fluid described in US patent 515,033 they would have been filled to a depth of 5 cm—a not unreasonable level for surface growth of a fungus. The weight of these vessels must have posed difficulties in handling; with hindsight, there may have been unrecognized problems of lead toxicity.

Pure citrate was obtained but the operation was not a commercial success. Nevertheless, Wehmer deserves much credit for this first industrial use of fungi. Until that time, apart from the empirical processes used in brewing and vinegar manufacture, there was hardly any experience with

large-scale fermentations with pure cultures, with the recovery of a pure product from large volumes of culture fluid, or with contamination problems. Moreover, Avery's work on lactic acid may not have been known to Wehmer. The process may have failed because ''it required too long a time on the technical scale'' (Currie, 1917). Wehmer later became a consultant to Bohringer for lactate production (Benninga, 1990) and tried to interest this company in the citrate fermentation.

A further US patent (1,066,358) for citrate production from sugar using ''*Sterigmatocystis nigra*'' was issued to B. Zahorski in 1913. Although Zahorski believed that his organism was not an *Aspergillus*, *S. nigra* is now recognized as a synonym for *Aspergillus niger*. It appears that the Zahorski process was never operated on the industrial scale.

Extensive studies by Currie showed that *A. niger* tolerated initial pH values of 2.5–3.5 and that extensive citric acid formation further lowered the pH (Currie, 1917). Much of Currie's work involved surface fungal cultures in Erlenmeyer flasks; shallow pans containing 1 liter of a 15% sucrose solution were also used (photographs of the pans are included in his paper but the specific dimensions are not given). Under these conditions, he obtained filtrates containing 10% of citric acid.

The rest of the citrate story might almost be encompassed with a single name—Pfizer. The saga of Charles Pfizer's fine chemical business, 1894–1977, has been recounted by S. Mines and our account is based on his history (Mines, 1978). The company, originally located in Williamsburg, Brooklyn, manufactured citric acid from crude citrate of lime and concentrated lemon juice from Italy and Sicily. By 1913, the monthly production of citric acid produced by the traditional process was 70,000 lbs. In that year, Currie contacted Pfizer about his revolutionary fermentation process with *A. niger*. Currie joined the company on May 15, 1917, working full time or as a consultant for the next 21 years.

The industrial citric acid fermentation introduced by Currie utilized pans of culture fluid. Contamination was a recurring problem, as it had been earlier for Wehmer. A pilot plant involving 8000 gallon batches went into operation in 1919. There was ''seesaw'' progress in dealing with contamination and the process required ''acres of pans'' (Christensen, 1951). Nevertheless, from April 19 to July 23, 1919, 100,000 lbs of calcium citrate were produced. Progress was regarded as satisfactory and a new building was constructed and named SUCIAC, Sugar Under Conversion Into Acid Citric. By 1923, 4500 lbs of calcium citrate were produced each day. With further expansions of capacity and refinements in the fermentation process, the cost of this commodity chemical declined from $1.25 per lb in 1919 to $0.20 per lb in 1939. In 1934, molasses from Poland was introduced as the carbohydrate substrate but this supply was eventually cut off by World War II.

Pfizer expanded into the United Kingdom in the early 1930s and a UK fermentation plant for citric acid was constructed by a team from the Brooklyn plant. Surprisingly, it performed better than did the one in Brooklyn. Mines' history indicates that the reasons for the higher yield were discovered and the same process was introduced at Brooklyn. The ''secret'' has apparently never been revealed—however, each member of the team received a special bonus of $2000. The Pfizer citrate operation continued for decades but was eventually taken over by Archer, Daniels, Midland during the 1990s (Magnuson and Lasure, 2004).

Research on the *A. niger* fermentation was also carried out in Germany at the beginning of World War I (Wehmer, 1933). The production strain used was the same one that Wehmer had studied for oxalate formation. The previously used factory at Thann was not available, so the work was carried out at the Chemical Factory in Winkel (near Wiesbaden) previously named as Goldenberg, Geronmont & Co. Although Wehmer had been promised reports of this work, they did not materialize. He noted ''that in war-time the work may have fallen asleep.'' The plan was to use a deep layer of medium ''with movement of the fluid'' (unter Bewegung der Flüssigkeit). It is not clear whether a full-scale shake process was under consideration (see later).

IV. SUBMERGED CULTURES

A. An interlude

By the late 1930s, industrial fermentations using surface cultures of *A. niger* were flourishing and profitable in both the United States and United Kingdom. Many tons of citric acid had been produced by surface fermentation and the Italian production from low-grade lemons ground to a halt around 1922. Although equipment for large-scale surface growth was unwieldy and it was hard to maintain sterile conditions, citric acid producers continued to use the surface culture method. ''Not until modern techniques of antibiotic fermentation were fully established did the citric acid producers begin to realize that they had stumbled up a cul-de-sac, and had almost the whole fermentation problem to tackle again if they were to make real progress'' (Hastings, 1971). Surprisingly, the long decades of development of the industrial process for production of citrate had little influence on the development of large-scale penicillin fermentations—it was the other way round.

Mycelial mats formed by surface growth are esthetically pleasing, as the following description of growth of the penicillin-producing *Penicillium notatum* B 592 attests: ''After 2–3 days the surface of the liquid in the

flasks became covered with a white felt; this gradually formed folds, the crests of which were blue-green; orange-yellow transpiration drops accumulated in these folds. The reverse side was golden yellow in color, and the color of the solution gradually deepened to an intense yellow'' (Clayton *et al*., 1944).

In the microbiological or biochemical sense, however, a mycelial mat floating on the top of a liquid is a very complex construction. The upper layers, exposed to air, are mostly dry and may be covered with spores; transpiration droplets may also be present. The lower layers are waterlogged and in contact with the culture fluid but have little access to air. To study the factors involved in fermentation, a more uniform cellular preparation is desirable.

The production of citric acid by *Aspergillus japonicus* using submerged culture had been reported (Amelung, 1930). A little later, working in Delft, The Netherlands, A. J. Kluyver, in collaboration with his student L. H. C. Perquin, began detailed and now classical studies using the ''shake-culture method'' to procure uniform cellular material (Kluyver, 1956; Kluyver and Perquin, 1933a). It was found that fungal growth under completely submerged conditions, with agitation or forced aeration, led to the formation of discrete, homogeneous, bead-shaped particles. In submerged cultures, cells are exposed to more uniform environmental factors (physical and chemical) during the growth process and yield a relatively uniform cellular preparation. Moreover, depending on the composition of the growth medium, considerable differences in metabolism occurred. This was not exactly a new concept, since Pasteur had aerated cultures of *Aspergillus glaucus* in 1876 and observed that fungal yield under aeration was almost six times that of growth in a closed flask (Foster, 1949).

Perquin's thesis (unfortunately for most of us written in the Dutch language) described production of oxalate and citrate by *A. niger*. Thus, to take a single example, using ''resting'' mycelium of *A. niger* prepared by submerged growth, the nitrogen source influenced the conversion of glucose to gluconic acid. Using ''basal medium I'' plus $NaNO_3$ gave a 100% conversion, whereas the same medium with $(NH_4)_2SO_4$ as nitrogen source produced only CO_2 (plus mycelium). For citric acid formation in ''basic medium II'' and using sucrose as substrate, the nitrogen source was again an important factor; the addition of NH_4Cl and a trace of $ZnSO_4$ gave a 39% conversion of sucrose to citric acid and a 61% conversion to CO_2 plus mycelium. When ''basic medium II'' contained NH_4NO_3 as nitrogen source, and an additional amount of KH_2PO_4, there was no formation whatsoever of citric acid and a 100% conversion to CO_2 and mycelium (Kluyver, 1956).

A second paper by Kluyver and Perquin (1933b) was concerned exclusively with kojic acid formation by *Aspergillus flavus*. When shake

mycelium was obtained by growth on malt extract medium (''Malzmycel'') subsequent replacement produced no kojic acid. Optimal conditions were the use of mineral media with a limited amount of NH_4NO_3 to prepare the homogeneous mycelium. Subsequent replacement then gave a 60–65% theoretical yield of kojic acid. Unhappily, the German occupation of The Netherlands ensured that Kluyver was unable to use his extensive knowledge of submerged fermentation in connection with the development of the antibiotic industry during World War II. However, one of his colleagues introduced the concept to researchers at Merck during the development of large scale penicillin production and Kluyver continued to contribute to the clandestine wartime Dutch research that later allowed Gist-Brocades to become a major antibiotic producer (Burns *et al*., 2002; Burns and van Dijck, 2002).

By 1949, the submerged culture method had been applied to a number of fermentations including those producing penicillin, ethanol, and various organic acids (citric, itaconic, kojic, and so on). With the significant exception of penicillin, these were laboratory and not full-scale industrial operations. Details of submerged culture techniques have been reviewed by Brown (1988).

B. Citric acid—the Wisconsin submerged process

In 1949, when penicillin was being produced in large amounts by submerged fermentations, Jackson Foster wrote that ''all mycological production (i.e., of citric acid) still is by the shallow pan surface process although recent patents make it likely that a shift to the more popular submerged method is imminent within a few years'' (Foster, 1949). In the same year, David Perlman noted some difficulties in citrate production in submerged cultures, but stated that ''... recent high production yields indicate that perhaps the major problems have been solved'' (Perlman, 1949). Interestingly, Pfizer had constructed a large, new plant in Groton, CT, that began citric acid shipments in April 1948—presumably this was still a surface operation.

Beginning in 1947, the cultural parameters that were important for submerged formation of citric acid with *A. niger* were investigated in detail (Shu and Johnson, 1947, 1948). Optimal compositions not only for the fermentation itself, but also for the medium used to derive spores for inoculations were determined. Using shake flask technology under optimal conditions, they obtained a yield of 70 g of citric acid per 100 g of added sugar. This yield compared favorably with those from the long-established surface fermentations. Further industrial research based on these observations provided a workable commercial scale technology for submerged fermentation. By 1953, all citric acid production for Pfizer

was ''in tanks'' (Mines, 1978). The biochemical and genetic aspects of industrial scale citric acid formation have been extensively detailed by Magnuson and Lasure (2004).

It had required some three decades from the time of Wehmer's patents to achieve success in commercial production of citric acid by surface fermentation. Almost three more decades were required to move to the industrial submerged process. Essentially, it required 60 years to proceed from the concept—produce a commodity chemical by fermentation—to large-scale successful production of citric acid with submerged cultures. The contrast with the approximately 5 years required to develop commercial scale penicillin production is marked.

V. WORLD WAR I SPURS FERMENTATION TECHNOLOGY TO PRODUCE GLYCEROL AND ACETONE

In Germany and Great Britain, during World War I, shortages occurred for starting materials required for explosives manufacture. In Germany, glycerol for the formation of nitroglycerine was in short supply while in Britain the shortage concerned acetone used as a solvent in the manufacture of cordite, a naval explosive.

A. Glycerol

The formation of small amounts of by-products during alcoholic fermentation with yeast was observed by Pasteur. In June of 1858, he noted that in ''one litre of wine there are several grammes of that product (i.e., glycerine, glycerol) which had not been suspected'' (Vallery-Radot, 1960, p. 85). It eventually was discovered that the yeast fermentation could be directed to the production of significant amounts of glycerol, and the appropriate manipulation of culture conditions was intensively investigated in Germany during World War I. Two workers, W. Connstein and K. Lüdecke, at the ''Vereingte Chemische Werke'' (Charlottenburg) investigated how changing the pH of the yeast fermentation might influence the overall metabolism leading to formation of products other than ethanol and CO_2. Under alkaline conditions, formation of these materials was decreased and that of acetaldehyde and glycerol increased. Addition of sodium sulfite gave the highest yields of glycerol and acetaldehyde. Inhibition of alcoholic fermentation by this reagent had been observed previously in 1874 by Dumas (Fruton, 1972). From 1000 kg of sugar, under optimum conditions, the following were obtained: CO_2, 373–420 kg; ethanol, 234–300 kg; glycerol, 230–247 kg; and acetaldehyde, 50–127 kg (Benninga, 1990). There were losses of glycerol on purification so

that from 1000 kg of sugar, only 140–160 kg were finally obtained. Nevertheless, using large fermentors (up to 600 m^3) this directed fermentation to form ''Protol'' (*Pro*pane*t*ri*ol*), achieving a monthly production of 1100 tons of glycerol.

This glycerol process required sugar—a human food that was also in diminished supply. Per head of population, Protol production required about 6 kg of sugar per annum—about 16–17% of the average prewar sugar consumption (Beninga, 1990). To a modern-day writer, besieged by claims of the benefits of ''low carb'' diets, this reduced consumption of sugar enforced by the needs of the military does not seem too great a hardship!

At about the same time as Connstein and Lüdecke's work, Carl Neuberg and his colleagues investigated the actual biochemical details of the sulfite fermentation. This directed fermentation is often termed eponymously, ''Neuberg's second form of fermentation.'' Acetaldehyde, formed as a normal intermediate in the Embden–Meyerhof pathway, was trapped as an addition compound in the presence of sulfite with the stoichiometry roughly as follows:

$$C_6H_{12}O_6 + NaHSO_3 \rightarrow HOCH_2{-}CHOH{-}CH_2OH + CH_3{-}CHOH{-}SO_3Na + CO_2$$

As a result, acetaldehyde was no longer available for reduction with NADH (to produce ethanol). Instead, dihydroxyacetone phosphate replaced acetaldehyde as an electron acceptor; the so-formed glycerol phosphate was converted to glycerol by dephosphorylation.

Under alkaline conditions, some acetaldehyde underwent a dismutation reaction to ethanol and acetic acid:

$$2CH_3CHO \rightarrow CH_3COOH + CH_3CH_2OH$$

As before, acetaldehyde was not available as an electron acceptor and glycerol was formed. This so-called ''Neuberg's third form of fermentation'' corresponds approximately to the following stoichiometry:

$$2C_6H_{12}O_6 \rightarrow CH_3COOH + CH_3CH_2OH + 2HOCH_2{-}CHOH{-}CH_2OH + 2CO_2$$

Glycerol, like other fermentation processes, eventually faced stiff competition from chemical synthesis via petrochemicals. In recent years, with the enormous increase in oil prices, renewed attention has been given to glycerol fermentation. Osmotolerant yeasts producing high glycerol yields have been isolated (Wang *et al*., 2001). One strain, *Candida glycerinogenes*, has been ''genetically improved'' and gives a conversion efficiency of up to 58% with glycerol concentrations of 110–120 g/liter.

B. Acetone, butanol, ethanol

The best known bacterial fermentation put into operation during World War I was that forming acetone and butanol as well as some ethanol. It is sometimes termed the ABE fermentation and has been well reviewed (Häggstrom, 1985; Hastings, 1978; Jones and Woods, 1986; Awang *et al*., 1988). The ABE fermentation required the exploration of a new concept, that is, rigorous sterility of large volumes of culture medium for an anaerobic bacterium. This process is forever associated with the name of Chaim Weizmann (Rose, 1986), but it was Fernbach who first used an anaerobe to convert potatoes to amyl alcohol, acetone, and butanol at the Institut Pasteur, 1910–1911 (Benninga, 1990). Weizmann, trained in Germany and Switzerland as a chemist, had obtained a position with W. H. Perkin at the University of Manchester in 1904. He became interested in synthetic rubber, concluding that a supply of butanol was mandatory. Beginning in 1909, he spent vacations at the Institut Pasteur, Paris, learning microbiology, and working with Auguste Fernbach (Rose, 1986). Weizmann, Fernbach, Perkin, and an entrepreneur named Halford Strange formed an unhappy collaboration and attempted to produce fermentation butanol. There were violent disputes among all of them and there was little or no progress. The project has been termed ''a sad exercise in commercial greed and academic politics'' and Perkin eventually belittled butanol (butyl alcohol) as ''butyl futile'' (Rose, 1986). Perkin left Manchester for Oxford in 1912, and Weizmann was then appointed as Reader in Biochemistry.

With the advent of World War I, the UK demand for acetone, a solvent used in the manufacture of cordite, increased significantly. It had previously been imported from Germany, where it was made by dry distillation of calcium acetate derived from wood. Weizmann realized that the acetone produced by his butanol process, initially regarded as a by-product, could be of value. At some point, supposedly in 1912, he had isolated (or obtained—there is some dispute) an organism, termed *Clostridium acetobutylicum* Weizmann, that could ferment grain. Cereal starch was used as a substrate. Soon, the UK government essentially appointed Weizmzann as Czar of acetone production and provided him generously with equipment and facilities. By 1916, a production level of 1000 kg of acetone/week was realized. Another plant had also been set up using Fernbach's organism and potato mash but with only half a ton of production/week (Rose, 1986).

Under Weizmann's direction several plants operated successfully—he even took over the one using the Fernbach method. By February 1917, acetone production was 228 tons monthly. However, a problem developed similar to that in Germany concerning glycerol production—namely, a critical shortage of grain, also needed for human nutrition. In response, there was a national campaign to collect horse chestnuts

(''conkers''), largely by children, to provide one alternative substrate. Unfortunately, foaming made the conker fermentation unmanageable. Acetone plants were therefore constructed in Canada where grain was plentiful. As the United States was drawn into the war, a plant was also built in Terre Haute, Indiana—a center for ethanol fermentation from corn (Gabriel, 1928). In North America, 2563 tons of acetone was made in the first 11 months of 1917. As the war came to a halt, some of these wartime plants were closed as uneconomical. However, at Terre Haute, they continued in operation by Commercial Solvents Corporation (Gabriel, 1928).

Weizmann eventually received considerable sums of money from patents and royalty payment from the UK government; the facts are not clear and the events have been described as ''cloudy'' (Rose, 1986). Much of his abundant energy had been taken up by his advocacy of Zionism, and in 1917 his work for the Zionist cause had led to the famous Balfour Declaration ''... His Majesty's government views with favour the establishment in Palestine of a national home for the Jewish people, and will use their best endeavours to facilitate the achievement of this object ...''. The Declaration was not a specific reward from a grateful government but there can be no doubt that Weizmann's achievements in fermentation chemistry influenced public opinion in a positive direction. In 1949, he became the first President of the State of Israel. The unintended consequences of the Balfour Declaration remain with us today.

In whatever manner the long-term consequences of Weizmann's political activities are evaluated, there can be no doubt as to the significance of the consequences of his scientific and industrial activities. At the time of World War I there was ''literally not a single fermentation vessel in Britain suitable for the purpose'' (Hastings, 1971)—that is, of the growth on a large-scale of an anaerobic bacterium. Beer was brewed in vessels constructed of wood or slate, and attempts to modify them by attaching lids were a failure. Eventually, vessels of mild steel with a capacity of 200,000 L were constructed as cylinders with hemispherical tops and bottoms (or conical bottoms) that could be steam sterilized under pressure. Obtaining and maintaining sterile conditions and preparing sterile media were problems. Another challenge was to prepare large volumes of inocula and to carry out aseptic transfer. Still further difficulties came with product recovery. These obstacles were all overcome. It is reasonable to say that this process marked the beginning of the modern fermentation industry: an industrial scale, anaerobic microbial process, with the complete exclusion of any contaminants, had been carried out for the first time. At a later date, the achievement of the ABE process gave confidence to the fledgling antibiotic industry that large scale submerged fermentation under sterile conditions could be undertaken. (There were, of course, new problems to be solved).

During the 1950s and 1960s, fermentation butanol could not compete with petrochemical material but the oil crisis of the 1970s led to renewed interest in the ABE fermentation. Moreover, genetic engineering of *C. acetobutylicum* strains became feasible leading to the possibility of higher yields and altered proportions of the three solvents (Woods, 1995). One strain of *C. beijerinckii*, termed BA 101, obtained by mutagenesis, had a high level of amylolytic enzymes and was characterized by higher yields of butanol and acetone than the parent strain. Butanol production from corn apparently would be an economic proposition using this strain (Qureshi and Blaschek, 2001).

VI. GLUCONIC ACID, KOJIC ACID

Although fumaric, itaconic, kojic, and oxalic acids all have been considered or investigated for production by fermentation there has been relatively little industrial demand for them (Foster, 1949; Miall, 1978). Gluconic acid, however, finds uses in human and veterinary medicine and in textile printing; the lactone form is used as a sequestering agent. The chemistry required for the conversion of the pyranose form of D-glucose to D-glucono-1,5-lactone is a simple oxidation. It has been carried out with intact microorganisms and by chemical or enzymatic catalysis. In the latter case, the necessary enzyme (obtained from various microorganisms especially fungi of the genus *Aspergillus* and *Penicillium*) is glucose oxidase, EC 1.1.3.4, using FAD as a cofactor (at one time named glucose aerodehydrogenase):

$$\beta\text{-D-Glucopyranose} + O_2 \rightarrow \text{D-Glucono-1,5-lactone} + H_2O_2$$

Subsequently, hydrolysis gave D-gluconic acid:

$$\text{D-Glucono-1,5-lactone} + H_2O \rightarrow \text{D-Gluconic acid}$$

The formation of gluconic acid in culture filtrates of *A. niger* was observed in 1922 by M. Molliard and the biochemistry was investigated by K. Bernhauer in the late 1920s (Foster, 1949). It had been discovered earlier as a product of *Acetobacter aceti*. However, the first attempts to develop a commercial fermentation by O. E. May and collaborators used a strain of *Penicillium luteum-purpurogenum* var. *rubrisclerotium* in shallow, aluminum pans. Attention turned quickly to submerged cultures, initially with *P. chrysogenum* and later with the more efficient *A. niger* (strain No. 67) (Foster, 1949; Miall, 1978; Magnuson and Lasure, 2004). More recently, laboratory scale continuous fermentation using *Aureobasidium pullulans* has been described (Anastassiadis *et al*., 2003).

In recent years, large amounts of kojic acid have been produced by fermentation with *Aspergillus oryzae* since this metabolite inhibits

tyrosinase activity. Tyrosinase activity is required for the formation of skin melanin; hence kojic acid has found use, especially in Japan, as a skin lightening agent (Bentley, 2006).

VII. PENICILLIN

The famous antibiotic penicillin, once a rare drug, has become a commodity chemical. The story is worth retelling. In 1928, Fleming discovered that a ''contaminating mould,'' growing on plates of staphylococcal variants, had caused adjacent bacterial colonies to become transparent and to lyse. When the mold was grown in an unspecified ''broth'' (Fleming provided few details) the culture fluid ''acquired marked inhibitory, bactericidal and bacteriolytic properties to many of the more common pathogenic bacteria'' (Fleming, 1929). The contaminating mold resembled *Penicillium rubrum*, but was later identified as *P. notatum*. The production of antibacterial activity was ''not common to all moulds or to all types of penicillium'' (Fleming, 1929). Fleming's original observation actually required an unusual set of circumstances that have been painstakingly recovered (Hare, 1970).

Fleming was a man of few words. To avoid repetition of the rather cumbersome phrase, ''mould broth filtrate,'' he coined the name, penicillin for the active material, deriving it from the name of the fungus. Initially, therefore, this famous name referred to a complex solution remaining after fungal growth. It required another 15 years before the pure, crystalline antibiotic was isolated. Fleming's experiments were poorly described. The mold filtrate itself was obtained from underneath a surface mat of mycelium when the fungus was grown on 200 ml of ''broth'' in a 500 ml Erlenmeyer flask. Lack of toxicity was also demonstrated but the experimental conditions would have been hard to duplicate.

Existing biographies of Fleming have tended to be either too partisan or dismissive. A new, nonpolemical approach to Fleming's life and the history of penicillin has been published recently (Brown, 2004), while Bennett and Chung (2001) have addressed the way in which Alexander Fleming became a ''scientific superstar.''

A. The work of Harold Raistrick

In 1932, penicillin was reinvestigated by Harold Raistrick and his colleagues at the Department of Biochemistry, London School of Hygiene and Tropical Medicine (Clutterbuck *et al.*, 1932). Raistrick had become interested in the production of organic compounds by fermentation following research on the acetone–butanol fermentation (unpublished) and the

investigation of glycerol production at Nobel's Explosives Company in Ardeer, Scotland. At Ardeer, he had also developed a high temperature (65 °C) fermentation of pulped cotton cellulose that produced high purity methane at the rate of 2000 feet^3/week (also unpublished) (Birkinshaw, 1972; Bentley and Thomas, 1990). The use of a thermophile was a remarkable achievement for industrial microbiology and was well ahead of its time. At the London School of Hygiene and Tropical Medicine, he went on to discover a large number of fungal secondary metabolites that were described in a series of 117 papers, ''Studies in the Biochemistry of Microorganisms.'' Carefully identified pure cultures, coupled with the use of chemically-defined culture media and controlled culture conditions (e.g., pH, temperature) were the hallmark of his research; almost all of his work was with surface cultures. After his death, he was praised as ''one of the finest natural products chemists this country (i.e., the United Kingdom) has ever produced . . .'' (Chain, 1971).

At roughly the same time that Kluyver was developing the shake culture technique, Raistrick and his colleagues made very detailed carbon balance studies on 240 different fungi using stationary growth conditions. Data from genera both familiar (e.g., *Aspergillus*, *Penicillium*) and unfamiliar (e.g., *Rhacoodium*, *Stysanus*) were carefully recorded (Raistrick, 1931). The results are undeniably reproducible, yet they were all achieved with the very heterogeneous cell preparations of surface mycelia. Another factor contributing to the lack of interest in submerged fungal culture was that shaking machines were not commonly available until well after the end of World War II. In the 1950s, one of us constructed a simple shaker from ''bits and pieces'' that was used by Marian Bentley in the Department of Bacteriology, London School of Hygiene and Tropical Medicine, for the submerged culture of dermatophytes such as *Microsporum canis* and *Microsporum gypseum*. It moved at 95–100 cycles/min with a stroke of 9 cm (Bentley, 1953). Marian said that Raistrick was not a little surprised by this development, and moreover that he was astounded that this brash, American lady had a spectrophotometer and a Beckman pH meter! His department was still using pH papers.

Although Raistrick failed to isolate pure penicillin, he contributed significantly to the early days of penicillin research. In collaboration with the mycologist, Charles Thom, he identified Fleming's fungus as a ''strain closely related to *P. notatum* Westling in the *P. chrysogenum* Thom series'' (Clutterbuck *et al.*, 1932). The Fleming strain was unusual since the type strains of *P. notatum* and *P. chrysogenum* did not produce penicillin. Rather than using Fleming's ill defined ''nutrient broth'' for growth, Raistrick showed that the organism grew well and produced penicillin on a chemically defined, ''modified Czapek–Dox medium.'' The fungi were cultured in one hundred 1-liter conical flasks containing 400 ml of medium. All strains that were examined produced a yellow pigment,

chrysogenin, and an alkali-soluble protein. Fleming's observations with respect to the antibacterial spectrum of penicillin were confirmed and Raistrick's group attempted to recover a purified antibacterial product. After removal of pigment and protein, the culture fluid could be concentrated by vacuum evaporation at pH 5.5 with retention of antibacterial activity; evaporation at other pH values resulted in loss of antibacterial activity. Most importantly, Raistrick's group showed that the activity could be extracted into ether from acid solution, indicating that penicillin was an acid. However, after removal of the ether in a current of air, the residue was inactive. On the other hand, if the ether extracts were evaporated with water *in vacuo* at 40–45 °C, the aqueous phase (after adjusting volume and pH) showed considerable activity.

In summary, Raistrick and his colleagues correctly identified Fleming's organism and showed that it was unusual in producing penicillin. They produced penicillin on a chemically defined medium, demonstrated that penicillin was an acidic material, and found that it was rather unstable.

Perhaps Raistrick showed a lack of imagination. Trained as a classical organic chemist, he predicted he would recover an active product from ether solution by evaporation; chemists of his era expected this extraction process to yield a solid product and sometimes even one that was already crystalline (a badge of purity). Penicillin confounded him by disappearing (i.e., losing its activity) during this mild operation. He was accustomed to handling more robust products than this willful biological activity. Had he only performed a simple back-extraction from ether into a buffered solution, the world might have had penicillin therapy in the 1930s rather than the 1940s and Raistrick would have crowned his career with a Nobel Prize. As the poet Whittier tells us:

> For all sad words of tongue or pen,
> The saddest are these: ''It might have been!''

He did not pursue further work on penicillin, in part because of difficulties in performing large numbers of bioassays, and perhaps, more persuasively, by the fact that medical colleagues advised that such an unstable material ''would never be of practical use in clinical medicine'' (Birkinshaw, 1972). Another factor may have been the low level of entrepreneurial spirit in UK universities during the 1930s and 1940s. Despite his industrial background, Raistrick lacked the drive and initiative that later powered Chain, nor was he a physician like Florey. The prevailing ethos among chemists of the time was that research should be done for its intrinsic interest and for the benefit of humanity, but not as a source of profit for the investigator. Famously, despite much urging by Chain, Florey refused to obtain any patents for penicillin. When patents later were obtained by individuals and pharmaceutical companies in the

United States, it became a matter of concern and dismay in the United Kingdom during the postwar years (Williams, 1984, pp. 309–314).

During World War II, Raistrick had an important role in organizing penicillin production in the United Kingdom. According to one writer, he ''rendered immense services'' for the General Committee of Penicillin in the United Kingdom and visited the United States to study penicillin progress there (Maurois, 1959). After World War II, he investigated possible developments in antibiotic production in Germany. His major influence in terms of industrial microbiology was to show that not only must a fermentation produce a useful product or activity, it was also necessary that the product or activity could be isolated and handled. Moreover, he demonstrated convincingly that microorganisms were potent sources for many compounds with varied chemical structures.

B. The development of penicillin at Oxford

There is little to be added to this oft-told story. Briefly, in a literature search for an antibacterial material, E. Chain and H. W. Florey, University of Oxford, located the published work of both Fleming and Raistrick. With the aid of an interdisciplinary team, they took over where Raistrick had left off. They used his chemically defined medium, but added yeast extract to speed up fungal growth. The project was initiated and planned by Florey, a physician who could see the enormous clinical potential. The major chemical work was carried out by Chain, a biochemist, and E. P. Abraham, an organic chemist. N. G. Heatley, a biochemist, devised the essential assay and supervised production. Most biological tests were the responsibility of Florey and M. A. Jennings, a physician. Bacteriological work and observations on the fungus were done by A. D. Gardner. C. M. Fletcher (yet another physician) had responsibility for human tests. All of these individuals were coauthors of the landmark 1940 and1941 papers (Chain *et al*., 1940; Abraham *et al*., 1941). The authors acknowledged the efforts of 10 (named) technicians ''without whose efforts adequate supplies of penicillin could not have been produced'' but who did not share authorship. In contrast, Raistrick's group consisted of three coauthors.

The major accomplishment of Florey's group was to prepare sufficient semipure penicillin to validate its clinical efficacy in humans. They carried out a classical ''scale up'' from laboratory level production to that of a small pilot plant. Their initial objective was to produce about 500 liters of ''mould filtrate juice'' per week. It is heart breaking to look back on the problems encountered in 1940 and 1941 in meeting this objective. Everyday living in the United Kingdom was then complicated by war-time shortages. Clothing, food, and petrol were all rationed. Shortages extended to laboratory equipment, reagents, and solvents.

Feeding experimental animals was difficult and the Medical Research Council had to supply Florey with a letter to the local War Agricultural Committee to maintain supplies (Williams, 1984, p. 155). Industrial personnel could be of little help in manufacturing penicillin since they too faced the same shortages and sometimes destruction of their facilities. Each evening, a rigidly enforced ''black out'' was imposed, and in some cities, smudge pots provided an unpleasant smoke screen. Bombing caused immense devastation in London and elsewhere. Transport was disrupted. Above all, there was the overarching threat of imminent invasion.

All members of Florey's team deserve our praise and gratitude, but for the success in scale up and pilot plant construction and operation, one man must be singled out—Norman G. Heatley, deceased, January, 2004, at the age of 92. For an appreciation of his life and work, see Moberg (1991). He had studied biochemistry at Cambridge and was awarded a Rockefeller Fellowship to work with the distinguished Linderstrøm-Lang in Denmark. Despite Denmark's neutrality it seemed unwise for him to go there so he declined the Fellowship and became a personal assistant to Florey. Heatley had many talents—''He was a most versatile, ingenious, and skilled laboratory engineer on any scale, large or minute. To his training in biology and biochemistry, he could add the technical skills of optics, glass- and metal-working, plumbing, carpentry, and as much electrical work as was needed in those preelectronic days. Above all, he could improvise . . .'' (Macfarlane, 1979, p. 302). To the supposed British talent for ''muddling through,'' he brought a superior level of skill and competence.

Vessels that could provide a large surface area of culture fluid for fungal growth, and also be readily sterilized, were in short supply and industry was unable to supply large numbers of any kind of glass container without lengthy delays. All manner of containers was put to use. Bed pans worked particularly well. Heatley recollected the use of slip-casting to make ceramic materials, and together with Florey, designed a container, roughly based on the shape of a bed pan. Florey contacted the pottery firm of James Macintyre and Company, Burslem, Staffordshire, who quickly made models and committed to producing 600 containers at a cost of £300. The containers were rectangular boxes, $27.5 \times 22 \times 6$ cm, with a sloping spout, internal diameter, 3.7 cm, holding a liter of culture fluid providing a depth of 1.7 cm. They were eventually termed ''jerries'' by Florey (''jerry'' is a slang term for a chamber pot).

By late December of 1940, the first batch of ceramic containers was ready. Two days before Christmas, Heatley drove a van, borrowed from the Emergency Health Service, the 100 miles from Oxford to Burslem (a little north of Stoke on Trent), to collect them, apparently unaccompanied. The van was unheated and the roads were perilous with black ice and piles of snow (Bickel, 1972, p. 112–115). To complicate matters, all

traffic signs and names of towns had been removed to frustrate the expected invaders. Even today, this is not a particularly easy trip. The M5 motorway (which did not exist in 1940) now passes close to Stoke on Trent and can be accessed near Cheltenham from Oxford by A40—a trip of about 40 miles. Heatley, however, is likely to have taken A34 passing through Stratford on Avon, Birmingham, and Stafford. Under unpleasant conditions, going slowly so as not to break any of the precious containers, the round trip required 11 h. He subsequently repeated the trip twice, ''once in a blinding snow storm'' (Bickel, 1972, p. 112–115) collecting the promised 600. The first batch of jerries was quickly unloaded and they were washed, sterilized, and inoculated on Christmas Eve and Christmas Day, 1940.

Heatley's daunting trips were the prelude to ''Florey's first step in turning his laboratories—with all their prized reputation as a world-class research centre—into a makeshift penicillin-producing factory'' (Bickel, 1972, p. 112–115). They deserve a larger place in the saga of penicillin development. The shift from Raistrick's use of the available 1-liter Erlenmeyer flasks (holding 400 ml of fluid) to a specially designed container (with a 1 liter capacity) is notable.

The firm of James Macintyre and Company also deserves more acclaim. They moved speedily and with skill to fabricate the jerries. This firm dated from 1843 when W. S. Kennedy had established a pottery business at the Washington Works at Burslem (one of the Five Towns immortalized as Bursley in the writing of Arnold Bennett). Kennedy was later joined by James Macintyre who had sole responsibility for the business. From 1860 onwards, the company was called James Macintyre and Company. An art pottery department was closed in 1913—at that time, the designer, William Moorcroft, left to set up his own factory subsequently earning fame for his line of Moorcroft pottery. From 1928, Macintyre produced only industrial ceramics such as electrical insulators, and of course, the now famous jerries. Today, items of Macintyre and Moorcroft art pottery command very high prices on the Internet.

But we digress! Let us return to Heatley's under-appreciated contributions. Not only did he contribute to the design of the ceramic pots and was responsible for their heroic transport, but he also solved two other major problems. The first was to devise equipment for a routine, easily accomplished bioassay of penicillin. To replace the original serial dilution assay method of Fleming for penicillin, which could only be applied to sterile material, Heatley devised a ''cylinder plate'' bioassay (Abraham *et al*., 1941; Heatley, 1944). Nutrient agar plates were seeded with a broth culture of *Stapylococcus aureus* by flooding and, after draining off excess broth, were ''dried'' in an incubator at 37 °C for 1 h. Short lengths (9.6 mm) of glass tubing (internal diameter, 5.1 mm) with an internal

bevel were placed on the agar plates and filled with the solution to be assayed. After 12–16 h incubation at 37 °C, each cylinder was surrounded by a circular zone where there was no bacterial growth. The diameter of the growth-free circle was measured using a glass scale illuminated from below. An arbitrary standard solution gave an average growth-free circle of 24 mm diameter. The amount of penicillin, dissolved in 1 ml of water, giving the same inhibition, was defined as one unit of activity—the ''Oxford unit.'' In early tests, the penicillin preparations contained from 40 to 50 Oxford units/mg (Abraham *et al*., 1941). It is clear that very small amounts of penicillin were involved since it is now known that 0.6 μg of the pure sodium salt of benzylpenicillin is equivalent to 1 Oxford unit (Coghill, 1998). In addition to cylinders of glass, porcelain cylinders, colored at the nonbevelled end to facilitate orientation, were also used. These ceramic cylinders were another contribution of the Macintyre Company. An extensive discussion of the microbiological and other assay methods for penicillin has been given by Scudi and Woodruff (1949).

Heatley's second contribution was a pilot plant to isolate the antibiotic. Acetone, citric acid, ethanol, and lactic acid and so on, all had been easily recovered from fermentation broths. The case of penicillin was vastly different. For the first time, it was necessary to deal with large volumes of crude filtrates containing an unstable material in low concentration. In much of the early work, the penicillin content of the filtrates was as little as 2 mg/liter. Raistrick had observed that it could be extracted into organic solvents such as ether from acidic solution. The Oxford group, however, replaced the dangerous solvent, ether, with amyl acetate. Clearly, the extraction of hundreds of liters of filtrate required some mechanization. Heatley rose to the challenge by devising a counter current approach and improvising the necessary equipment. The cold filtrate was acidified with phosphoric acid at the last moment (penicillin is unstable as free acid in aqueous solution), which then fell in tiny drops through an up-flowing column of amyl acetate. An amyl acetate solution of penicillin was withdrawn at the top; exhausted filtrate was discharged at the bottom. Heatley made six units for this counter current extraction apparatus. ''It was an effective and continuous process, but one involving a maze of glass tubing, junctions, pumps, cooling coils, warning bells, or lights, and much associated plumbing. It was a creation that would have appealed to Mr. W. Heath-Robinson—and it worked'' (Macfarlane, 1979, p. 307). The extracted penicillin was recovered by reversing the same process using a slightly alkaline-buffered aqueous solution. Finally, lyophilization gave a fairly stable, brown powder.

Chain, an experienced biochemist, surprisingly felt that back extraction of penicillin from an organic solvent was unsound. Perhaps he was

annoyed that Heatley had suggested the approach in a conference with Florey from which Chain was excluded. It is recorded that he challenged Heatley to go and do it as ''that will surely be the best and quickest way to show that you are wrong'' (Bickel, 1972, p. 91). Fortunately for humanity, Heatley was right.

In summary, Florey's group had shown that a labor-intensive and improvised pilot plant could produce penicillin—albeit impure—that was effective in treating infections. Key factors were an efficient bioassay, design, and production of a ceramic pot holding 1 liter of culture fluid, and use of a counter current extraction process coupled with lyophilization to produce a stable product.

C. The experience in the Unites States and development of submerged fermentation

By mid 1941, it was clear to Florey and to Sir Edward Mellanby, the Executive Secretary of the all important Medical Research Council, that further progress in producing penicillin in the United Kingdom was going to be painfully slow. It was decided that only in North America, particularly the United States, could adequate production facilities be put in place. Florey and Heatley, therefore, traveled to New York, initially by the very dangerous air corridor to neutral Lisbon, and from there by the Clipper service to New York.

The early penicillin story in the United States had two components. The first was a remarkable, small-scale duplication of the Oxford group's methods in New York. The second was the massive scale up of penicillin production using submerged fermentation by a consortium of government and industrial laboratories.

At Columbia University's College of Physicians and Surgeons, New York City, Dr. Martin Henry Dawson had sought for some time a treatment for subacute bacterial endocarditis. Soon after the August 24, 1940 issue of Lancet, with its news of penicillin, had been received in the United States, Dawson together with Gladys Hobby, a microbiologist, and Karl Meyer, a biochemist, decided ''to make some penicillin'' (Hobby, 1985). Following the methods developed at Oxford, they inoculated hundreds of 2 liter flasks. Less than three months later, on October 15, 1940, penicillin was administered parenterally to two patients at Presbyterian Hospital (New York City). The amount of penicillin was too small to provide a therapeutic response, but the low toxicity of the crude preparations was notable. This small but dedicated group, using the information freely published by Florey, actually succeeded in using penicillin parenterally before the Oxford group.

D. Submerged fermentation for penicillin

Although Florey and Heatley initially experienced some setbacks during their attempts to get penicillin production underway in the United States and Canada, they were largely successful. Several pharmaceutical manufacturers were interested and initially large ''bottle plants'' for surface production were constructed; in this early US work, no attempts were made to use the ceramic jerries. Heatley's stay at the Northern Regional Research Laboratory (NRRL) in Peoria, IL (July to November, 1941) was extremely productive. There, he demonstrated his Oxford methods and, in collaboration with the NRRL staff, found that a substantial increase in penicillin yield was obtained when the growth medium was amended with corn steep water (also termed corn steep liquor), a then useless by-product of the corn industry. Moreover, work was begun on submerged fermentations. A detailed, but very readable account has been written by Gladys Hobby a member of Dawson's team as just noted, and herself a major contributor (Hobby, 1985). She joined Pfizer in 1943 and ''played a major role in the company's successful entry into the antibiotics field'' (Mines, 1978). For a more comprehensive account, we refer readers to Hobby's excellent book and to other sources (Brown, 2004; Mateles, 1988).

The key steps that turned penicillin into a commodity chemical and, therefore, an economically feasible option for widespread therapeutic application, are summarized below:

- Increased yields by addition of precursor molecules (e.g., those in corn steep water) to growth media; detailed determination of optimal conditions for media compositions, pH, aeration, and time and temperature of the fermentation process.
- Selection of high yielding fungal strains. Although strain selection was not a new concept (it had already been used, e.g., with the citrate and gluconate fermentations) the scale of the search was unprecedented—strains of penicillin-producing fungi were obtained worldwide. Ironically, the high-yielding strain eventually selected for scale up was found in Peoria. Mary Hunt Stevens, a worker at NRRL, located so many fungal samples that she became known affectionately as ''Moldy Mary.'' Her triumph was to find a moldy cantaloupe at the Illinois Fruit and Vegetable Company, 533 Main Street, Peoria (Scoutaris, 1996). It yielded *Penicillium chrysogenum*, numbered as NRRL 1951. This organism produced good penicillin yields in submerged cultures. Eventually, even better results were obtained from mutated strains selected after X-ray or UV treatment and the action of mutagenic agents; it is the progenitor of most production strains in use today.

- Development of the appropriate equipment for industrial use—a task for chemical engineers—was made difficult by both lack of materials and of any consistent design criteria. Not only did the equipment for the fermentation tanks themselves have to be designed, but provision had to made for delivery of a sterile air supply. Additional equipment was needed for efficient antibiotic recovery (Mateles, 1998).

Today, the β-lactam antibiotics, primarily penicillins and cephalosporins, are indeed commodity chemicals. The major industrial producers now obtain yields of 40–50 g/liter for penicillin—orders of magnitude greater than the pathetic amounts from Florey's original pilot plant. Moreover, as productivity and recovery have increased, production costs have declined. For example, in 1953, the bulk cost for benzylpenicillin was ~$300 per kg; in the late 1990s it ranged from $10 to $ 20 per kg (Elander, 2003).

The end is not in sight. Increasingly, genetic techniques, including the deciphering of entire fungal genomes, are providing basic information about the genes and enzymes involved in antibiotic production. Moreover, solid state fermentation techniques are being modernized and have several advantages over submerged liquid cultures (Krishna, 2005). No commodity chemical has had such an exciting history as has penicillin, and, with the exception of ethanol, no other fermentation product has had so profound an effect on human lives. In the century and a quarter since Avery began lactic acid production, there have been far reaching developments involving biochemicals of various kinds. It is now clear that in many cases, a microorganism is an excellent industrial chemist.

In closing, it is appropriate to remember the ''Laws of Applied Microbiology'' formulated for a 1979 speech given at the Annual Meeting of the Society for Industrial Microbiology by the distinguished industrial microbiologist, David Perlman. Perlman insisted that these laws ''must be observed for success'' (Perlman, 1980).

1. The microorganism is {always right, your friend, a sensitive partner}.
2. There are *no* stupid microorganisms.
3. Microorganisms {can, will} do anything.
4. Microorganisms are {smarter, wiser, more energetic} than {chemists, engineers, and so on}.
5. If you take care of your microbial friends, they will take care of your future (and you will live happily ever after).

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CHAPTER 2

Submerged Culture Fermentation of "Higher Fungi": The Macrofungi

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I. INTRODUCTION

A. Definition of "higher fungi"

This review will critically evaluate recent advances in submerged liquid cultivation of the vegetative or mycelial forms of those members of the ''higher fungi'' that produce macroscopic spore-bearing structures, namely the macrofungi. This group of organisms has been rather neglected from a biotechnological viewpoint, when compared to the more widely exploited, and better understood microfungi. In part, this is due to the complexity and sophistication of their life cycles; so in order to understand the limitations and barriers to our future exploitation of these fungi, the taxonomy of these fungi and their life cycles will be briefly discussed first.

The taxonomy of the fungi is in a state of rapid flux arising from the widespread use of molecular genetics to characterize and classify these organisms, and many of our earlier ideas on their phylogenetic relationships must now be revised (Hibbett *et al.*, 2007). Fungi can be defined as being eukaryotic, characteristically mycelial organisms, with chitin-based cell walls, an absorptive nutrition, and which reproduce by production of spores (Deacon, 2005). Currently, five divisions are recognized, based on their sexual and asexual reproductive structures: Chytridiomycota, Zygomycota, Ascomycota, Basidiomycota, and a new phylum, the Glomeromycota (Walker and Schüβler, 2004), whose members were previously included in the Zygomycota. The major divisions of fungi are shown in Fig. 2.1 and some examples in Fig. 2.2.

Although the water molds and slime molds have always been considered as fungi, and are still studied by mycologists, it is now clear they are misplaced here. Neither possesses a chitin cell wall, but instead have galactosamine and cellulose walls, respectively (Carlisle *et al.*, 2001). They are now included among the algae and protozoa. A detailed account of fungal classification, species characteristics, habitat, morphology, and other factors of interest can be found on Web sites such as GenBank (www.ncbi.nlm.nih.gov/Taxonomy), the Tree of Life Web Project (www.tolweb.org). Relevant textbooks in this area include Carlile *et al.* (2001), Deacon (2005), Gow and Gadd (1995), Kirk *et al.* (2001), and the recent taxonomic reviews of Lutzoni *et al.* (2004), Spatafora *et al.* (2006), Taylor and Berbee (2006) and Hibbett *et al.* (2007) are also valuable.

The term ''higher fungus'' is, of course, a subjective descriptor, so it is important to define which organisms are included and to clarify their taxonomic status. According to Worgan (1968), Redhead (1997), and Deacon (2005), the higher fungi are those filamentous fungi that form conspicuous and fleshy fruiting bodies containing their sexual spores, whereas, the ''lower fungi'' are defined here as ''fungi whose

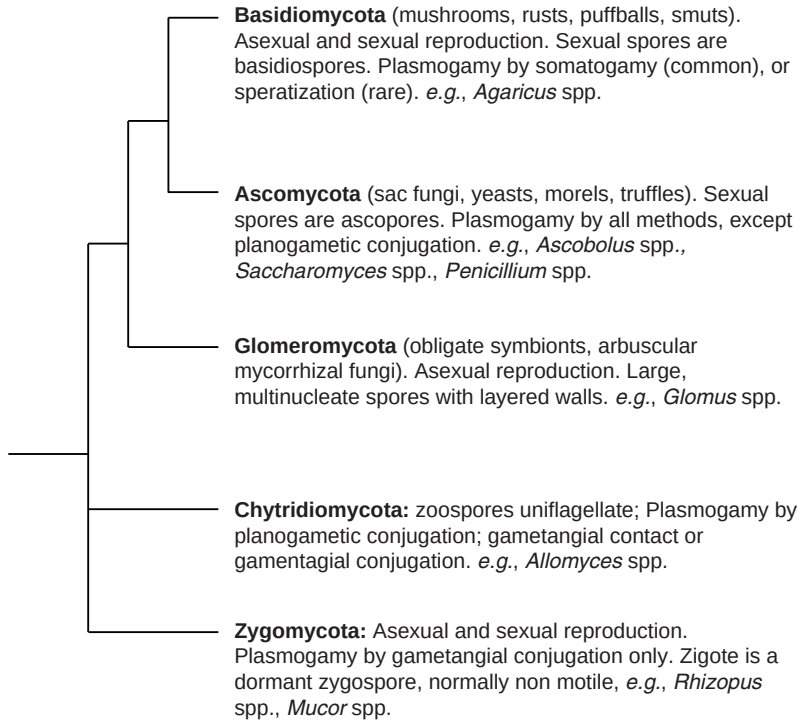

FIGURE 2.1 Phylogeny of true fungi (Eumycota), adapted from Deacon (2005) and Carlile et al. (2001).

characteristic nature is distinctly filamentous, but which do not form any organized specialized fleshy fruiting structures of macroscopic dimensions.'' In other words, according to this definition, the terms ''higher fungi'' and ''macrofungi'' are synonymous, and the term ''mold'' incorporates the ''lower fungi.'' It must be stated that there is no universal agreement on this method of grouping. Gow and Gadd (1995) and Carlile *et al.* (2001) distinguish ''higher fungi'' from ''lower fungi'' in terms of their hyphal and mycelial evolution and organization. So, using their criteria, ''higher fungi'' have septate–reticulate hyphae, in contrast to the coenocytic hyphae of the typical ''lower fungi,'' and often, but not always, produce fruiting bodies, meaning that the vast majority of the *Ascomycota* and *Basidiomycota* are included.

This review will focus on recent developments in the submerged culture of the vegetative or mycelial forms of the ''higher fungi'' producing fruiting bodies, which are described here as the macrofungi (most members of the *Ascomycota* and *Basidiomycota*), with particular emphasis placed on those producing potentially useful natural products and novel

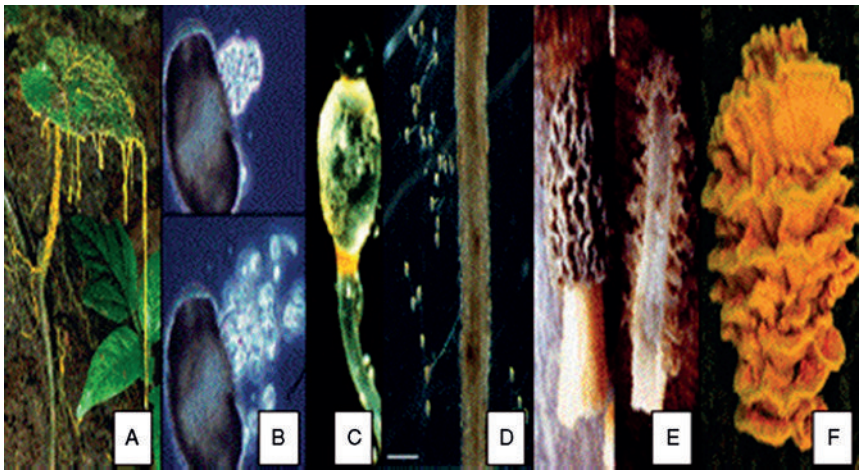

FIGURE 2.2 (A) Plasmodium of a slime mold dripping from a leaf in a rainforest. (B) Chytridium growing on a single pine pollen. (C) Pilobolus kleinii (zygomycota). (D) Roots of Plantago media (plantain) colonized with Glomus clarum. (E) Two fruiting bodies of the edible Morel (Morchella) with one slice open (Ascomycota). (F) Fruiting body of Laetiporus sulphureus (Basidiomycota), known as the chicken-of-the-woods. All photos taken from The Tree of Life Project (www.tolweb.org).

compounds with interesting biological activities by means of submerged culture fermentation processes. All other filamentous fungi that do not form macroscopic fruiting structures will be excluded, as these have been frequently, extensively reviewed and much more is understood about their genetics, physiology, and biotechnological potential (e.g., see Gibbs *et al.*, 2000). Interesting compounds with antibacterial, antifungal, phytotoxic, nematocidal, cytostatic, antiviral, and other pharmacological activities have been obtained from the macrofungi (Lorenzen and Anke, 1998), so these organisms are becoming of increasing interest, and this raises fundamental challenges in understanding how organisms with such complex life cycles can be transformed into a simple hyphal network in liquid culture inside a bioreactor. This is an area of fermentation technology that has not been properly discussed since the review of Eyal (1991). One example is the ''medicinal mushrooms,'' most of which belong to Basidiomycota, which have already gained interest worldwide, as they impact beneficially on our health (Manzi and Pizzoferrato, 2000). Most commercial products from these fungi are still produced by traditional basidiocarp production in logs or sawdust (solid-substrate fermentation (SSF)). These are slow processes which often take months (see Section II.A), where process control is limited, and standardization of the product cannot be guaranteed. However, submerged fermentation technology of

their vegetative forms in many has been achieved, and shows promise as an alternative because it has none of the weaknesses or limitations mentioned above.

B. General considerations

Some of the macrofungi are edible, and some also have biotechnological and environmental applications. Edible macrofungi have long been consumed both as valuable protein and energy sources, and also for improving human health and longevity. For example, *Pleurotus ostreatus* (Oyster mushroom), *Lentinus edodes* (Shiitake), *Flammulina velutipes* (Enokitake), Chanterelle, and *Agaricus* spp., are consumed worldwide. They are considered to be healthy, being both low in calories and fat, but rich in proteins, minerals, and dietary fiber (Manzi and Pizzoferrato, 2000). Of course, what constitutes a ''healthy'' food reflects a subjective opinion, since for much of its existence the bulk of humanity struggled to obtain sufficient nutrients to subsist upon. So the notion that a low calorie/low fat food is a healthy one is a modern concept indeed. The cell walls of edible macrofungi contain chitin, hemicelluloses, glucans, mannans, and especially, branched noncellulosic β-glucans, which are thought to have beneficial health properties. The biological activities of such polysaccharides specifically depend on the chemical backbone, the structure of the side chain groups, and the degree of branching (Chen and Seviour, 2007). Several polysaccharides, β-glucans, from macrofungi are used clinically in Japan and China, including schizophyllan, lentinan, and krestin. Their biological properties depend on the microorganism, the medium composition, and operational conditions, as discussed below.

In terms of biotechnological and environmental applications, the Basidiomycota, for example, contain several different ecological groups. Thus the white rot, brown rot, and leaf litter fungi obtain their nutrients in different ways (Songulashvili *et al.*, 2007). White rot fungi are the only organisms capable of degrading all basic wood polymers, and produce cellulases and hemicellulases and unique oxidative (ligninolytic) extracellular enzymes, which degrade cellulose, hemicellulose, and lignin into low-molecular-weight compounds that can be assimilated for nutrition. By contrast, the brown rot fungi only degrade cellulosic and hemicellulosic components of the wood.

Clearly, the manner in which macrofungi obtain their nutrients in nature may have an impact on how we seek to grow them in fermenters, and how we scale up desirable metabolic traits in liquid culture systems. Controlled SLF techniques can revolutionize their production of specific metabolites providing possibilities for scaling up and developing economically viable industrial processes.

The following lists a range of industrial applications of macrofungi that have been investigated with both submerged liquid, and more traditional solid-state fermentation cultivation techniques:

1. Production of mycelium of edible mushrooms for human and animal consumption as food, mushroom-flavoring agents, and natural aromatic flavors (Kuhad *et al.*, 1997; Lomascolo *et al.*, 1999).
2. Production of fermented food by fungi (Kim *et al.*, 2004; Mukhopadhyay *et al.*, 2005).
3. Production of spawn for the cultivation of fruiting bodies (Eyal, 1991; Kananen *et al.*, 2000).
4. Production of chemical specialties including water-soluble polysaccharides (Ooi and Liu, 1999), vitamins, enzymes (Rogalski *et al.*, 2006), organic acids (Eyal, 1991; Rau *et al.*, 1992; Wang and McNeil, 1995), and nucleotides.
5. Production of therapeutic compounds such as antibiotics (Gehrig *et al.*, 1998), anticancer agents (Daba and Ezeronye, 2003), and antifungal agents (Liu and Wang, 2007; Ngai and Ng, 2003); aflatoxin control (Zjalic *et al.*, 2006).
6. Application in processes aimed to recycle industrial and agricultural wastes (Adamovic *et al.*, 1998; Ambrósio and Campos-Takaki, 2004; Elissetche *et al.*, 2006; Fu and Viraraghavan, 2001; Lee *et al.*, 2003; Pal *et al.*, 1995; Reddy, 1995; Revankar and Lele, 2007; Shojaosadati *et al.*, 1999; Villas-Bôas *et al.*, 2002).

We will first briefly describe the life cycles of these organisms (Section I.C.), since an understanding of these is fundamental to a systematic approach to fermenter cultivation of the vegetative forms. Section II discusses in detail the influence of different process parameters and modes of cultivation on the growth and product formation by submerged cultures of macrofungi. Section III discusses recent studies on products and applications of macrofungal fermenter cultures, and finally, future developments in their exploitation are appraised.

C. Life cycles

Ascomycota and *Basidiomycota* are grouped into the subkingdom Dikarya (Hibbett *et al.*, 2007), reflecting the putative synapomorphy of their dikaryotic hyphae, proved by the similarity between the structures that coordinate the formation of the dikaryotic cells in both groups. Thus, Ascomycota form croziers and Basidiomycota form clamp connections (Fig. 2.3). However, Ascomycota usually have mycelia with simple tapered septa, whereas Basidiomycota have a complex dolipore septum, sometimes with clamp connections. In Basidiomycota, the dikaryotic

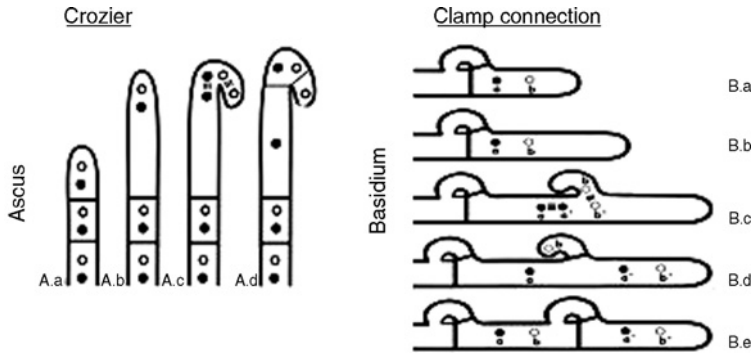

FIGURE 2.3 Mechanism for the formation of the dikaryon stage in Ascomycota (Crozier) and Basidiomycota (Clamp connection). Crozier formation: (A) a—Hyphal tip of ascogenous cell; b—Elongation of hyphal tip; c—Hyphal tip recurves forming crozier; d—Simultaneous division of nuclei and division of crozier into uninucleate apical and binucleate penultimate cells. Stalk cell below penultimate cell is also uninucleate. Clamp Connections in the Basidium: (B) a—Terminal cell of hypha with nuclei; b—Hyphal tip elongating; c—Synchronous division of nuclei and clamp branch attaches to hypha; d—Septum forms at base of the clamp forming new cell at hypha tip; e—Now both the terminal and subterminal cells are binucleate, each with a compatible pair of nuclei.

mycelium is the dominant form throughout the life cycle (Clark and Anderson, 2004), unlike transient dikaryotic cell persistence seen in Ascomycota life cycles. Their cells exist mainly as diploids, whereas Basidiomycota cells exist as dikaryons in which the two genetically compatible nuclei exist separately, dividing in a coordinated manner. In terms of their submerged liquid cultivation this feature has implications, as a complex and synchronized process like the formation of clamp connections and maintenance of the dikaryotic state, results in modest growth rates compared with other fungi, making the fermentation process of Basidiomycota generally longer than those of the Ascomycota. Finally, Ascomycota form their sexual spores, ascopores, inside an ascus, whereas in the Basidiomycota, exogeneous basidiospores are formed on a special sporogenous cell called a basidium (Carlile *et al.*, 2001). Table 2.1 summarizes the main differences and similarities between these.

1. The Ascomycota

The Ascomycota comprise about 60,000 described species, ~75% of all described fungi (Kirk *et al.*, 2001), including many economically important fungi. Most fungi that cause food spoilage belong to Ascomycota, which also includes several yeasts and *Neurospora*, which played an important role in the development of modern genetics. Many cause serious plant diseases, including powdery mildews that attack fruits,

TABLE 2.1 A Comparison of *Ascomycota* and *Basidiomycota* features

	Differences		
Shared features		Ascomycota	Basidiomycota
Cross-walls (septa)	Meiospores	Four endogenous (ascopores)	Eight exogenous (basidiospores)
Chitin cell walls with glucans	Dominant phase	Homokaryon	Dikaryon
Dikaryotic stage	Dikaryon formation	Crozier	Clamp connection
Sexual, asexual reproduction	Plasmogamy methods	All except Planogametic conjugation	Somantogamy (common) Speratization (rare)

chestnut blight, and Dutch Elm disease caused by *Ceratocystis ulmi*, a fungus native to certain European countries. The edible morels and truffles are also Ascomycota, producing a range of macroscopic fruiting bodies, and therefore are considered here as macrofungi.

In general, a typical sexual life cycle involves plasmogamy most commonly by the method of gametangial contact where a male gametangium or antheridium contact ''female'' gametangium or ascogonium leading eventually to production of one or more asci. Then, the two haploid nuclei fuse (karyogamy), and the diploid nucleus immediately undergoes meiosis, resulting in four genetically unique haploid nuclei that are incorporated into spores. One mitotic division usually follows meiosis so that most have eight-spored asci. The typical life cycle is represented in Fig. 2.4.

2. The Basidiomycota

The Basidiomycota currently comprise about 30,000 species, which is 37% of the described species of fungi (Kirk *et al.*, 2001). These are of ecological and industrial importance. Some are saprobes feeding on dead or decaying organic material or wastes, and these play a critical role in recycling organic material because of their ability to degrade complex (bio)molecules, especially those recalcitrant to biodegradation by other groups of microbes. Some are symbionts, forming mycorrhizae with many vascular plants, which facilitate nutrient assimilation. Others are fungal, plant, or animal pathogens responsible for serious crop losses (*Ustilago maydis*) or potentially fatal human diseases (*Cryptococcus neoformans*). Furthermore,

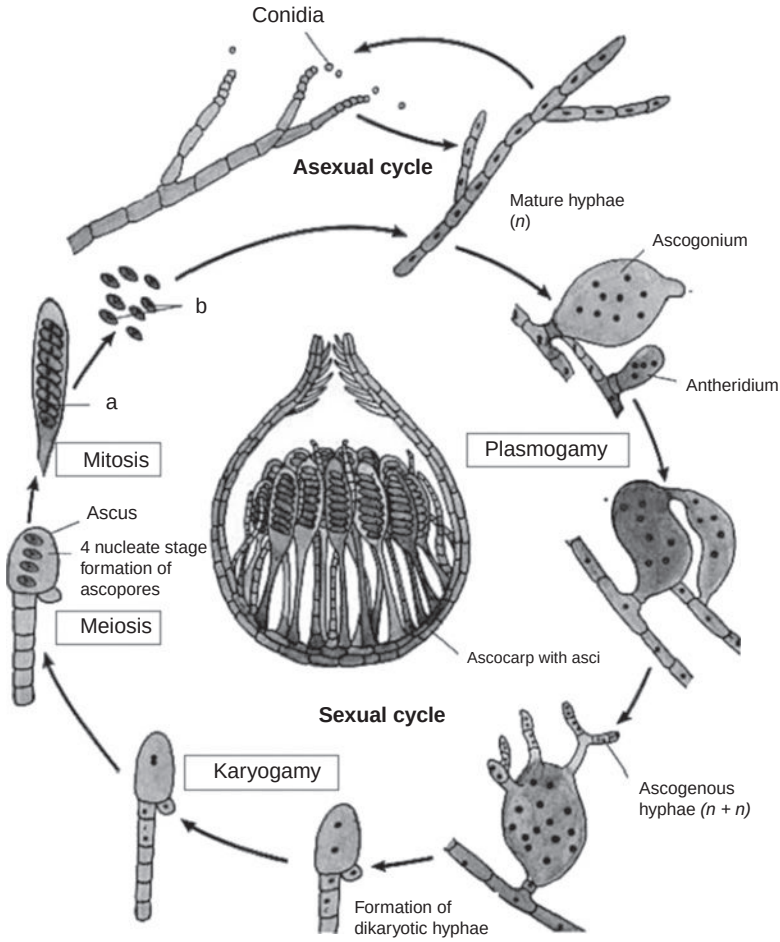

FIGURE 2.4 Life cycle of a typical ascomycete. Ascus development: a—Eight nucleate stage following mitosis; b—Eight ascospores in mature ascus.

the fruiting bodies of many species have been used traditionally as a source of food (*Boletus edulis*, *Lactarius* spp.), while some have hallucinogenic properties (*Amanita muscaria*, *Psilocybe* spp.), and others are deadly poisonous (*Amanita phalloides*).

The Basidiomycota life cycle starts with germination of a haploid basidiospore to form monokaryotic mycelia, where each cell contains a haploid nucleus (primary mycelium). When two genetically compatible monokaryons encounter one another, they carry out somatogamy to produce secondary mycelium consisting of dikaryotic cells. The two haploid nuclei divide synchronously by a mechanism involving clamp

connections, which are believed to function so as to ensure that the dikaryotic state of the cells is maintained (Deacon, 2005). All fungi that produce clamp connections are members of the Basidiomycota, but not all Basidiomycota produce clamp connections. Thus, in liquid cultures submerged hyphae may fail to form clamp connections, but the aerial hyphae form them abundantly (Carlisle *et al.*, 2001).

Basidiomycota spend most of their life cycles as dikaryons, but in response to environmental stimuli they eventually form a basidiocarp (spore-producing cells), the basidia (singular basidium) develop on the hymenial layer; then karyogamy occurs where the haploid nuclei fuses to form a diploid nucleus. This is followed by meiosis. Typically, four haploid daughter nuclei are produced, and each one then migrates into each of the basidiospores, which develop externally on the basidium. Each usually contains one or two of the haploid meiotic products (Griffin, 1994). From a structural viewpoint, the fruiting body (mushroom) is an efficient reproductive body for spore liberation and the cap acts as a protective shield.

Figure 2.5 shows the main features of the life cycle of Basidiomycota, such as *Coprinus fimetarius*, *Schizophyllum commune*, *Lentinus edodes*, *Fl. velutipes*, and *Pl. ostreatus* (Raper, 1978), producing fruiting bodies called ''mushrooms'' or ''toadstools.''

II. GROWTH IN SUBMERGED CULTURE

A. Solid-substrate fermentation *vs.* submerged liquid fermentation

SSF is characterized by the growth of filamentous fungi on a solid substrate (e.g., grain) in the near absence of free water ($a_w \approx 0$), but with sufficient presence to support their growth and metabolism (Mitchell and Lonsane, 1992). SSF often exploits agroindustrial residues as substrates, adding value to them. In general, fungal extracellular enzymes break down refractory biopolymers, like cellulose, which allows further microbial processing to yield valuable products. SSF has been around for a long time. For example, the Koji processes which produce soy sauce, *miso*, *sake*, and *tempeh*, are an ancient technology, as is the production of the composts and mushroom growing; and bioremediation and biodegradation of hazardous compounds, biological detoxification of agroindustrial residues, biotransformation of crops for nutritional enrichment, biopulping, production of some fungal secondary metabolites and enzymes, and biopharmaceuticals all use SSF (Carlisle *et al.*, 2001; Gonzalez *et al.*, 2003; Mukheriee and Nandi, 2004; Nwe *et al.*, 2001; Ngai and Ng, 2003). It is important to stress that the applications of SSF described earlier include all fungi, and not particularly macrofungi, as defined here. More information regarding

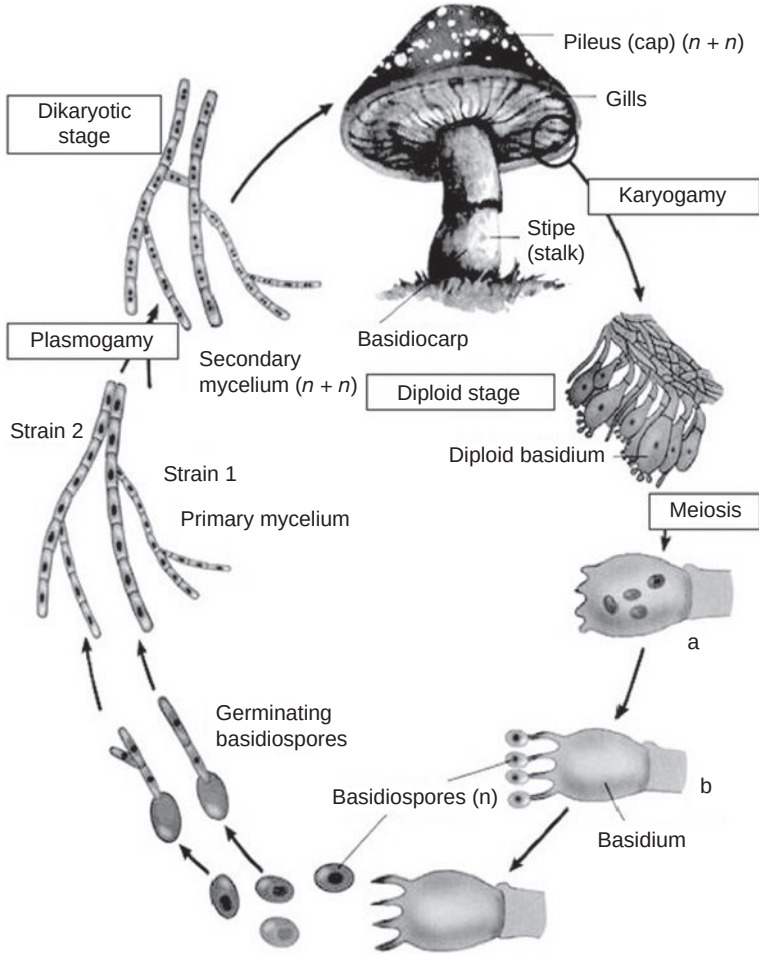

FIGURE 2.5 Life cycle of typical basidiomycete. Basidium development: a—Formation of basidiospores, but the 4 nuclei have not migrated into spores; b—Nuclear migration into basidiospores (Raper, 1978).

fundamental aspects of SSF is given by Mitchell and Lonsane (1990), Pandey *et al.* (2000), and Pandey (2003).

Even though almost all ancient fermentation processes were of SSF, SLF processes have largely replaced these for production of microbial metabolites and cells in developed economies. Consequently, SSF processes have been largely neglected as means for obtaining higher value products in western countries. This may have been because of the enormous impact the large-scale production of penicillin had during World War II, and subsequently, becoming the model technology for any fermentation.

However, over the last decades, there have been significant developments in biochemical fermentation engineering related to SSF (Pandey, 2003), and these have targeted many of the problems traditionally associated with SSF, including low O_2 transfer rates, CO_2 and heat removal, and bacterial contamination. Furthermore, improved process control via kinetic analysis and mathematical modeling, together with novel designs of bioreactors, has also improved the potential of SSF as a production technology (Suryanarayan, 2003).

SSF normally uses cheap raw materials, which do not need prior sterilization, it has relatively low power inputs, and waste disposal is comparatively straightforward. However, it is often a slow process, and is limited by the choice of organisms able to tolerate the low a_w levels involved. In addition, SSF is difficult to monitor, control, and scale-up, and is limited to processes that do not require ''containment'' (meaning that the operators can come into contact with the product during the cultivation). Thus, SLF has become the preferred method of fermentation for commercial applications. SLF is intrinsically less problematic (heat and oxygen mass transfer are much better, and culture homogeneity is usually superior) making it more reliable and reproducible, easier to monitor and to control key operational parameters, and more flexible.

However, SLF of macrofungi only began slowly during the 1950s, using principles shown to work with the lower fungi in fermenters (Eyal, 1991). The justification in this approach is clear. Macrofungi have been consumed for their flavor, nutritional value, and medicinal properties since ancient times, especially in Asian cultures. They were traditionally grown in composts, logs, and sawdust (by SSF), processes which take from one to several months before fruiting bodies appear. Furthermore, fruiting body composition is highly variable, especially in its content of biologically active substances, and this traditional method makes extraction, for example, of polysaccharides from fruiting bodies challenging (Lo *et al.*, 2006). SLF offers the potential to produce these compounds more rapidly and hygienically, and hence ensure their reliable supply not influenced by seasonal variations. Since the first description of mycelial growth of *Agaricus campestris* in a synthetic medium by submerged fermentation (Humfeld, 1948), numerous developments have been made to establish submerged cultivation processes for these fungi on an industrial scale. Macrofungi in fermenters grow slower than bacteria, and other filamentous fungi, making optimization and control in terms of liquid fermentation a challenge.

Thus, SSF or SLF is not interchangeable. Many factors, including economic considerations, product quality, and government regulations for disposal of wastes (solid or liquid), may help to determine the choice of the process to use.

B. Isolation and maintenance of the cultures

The first stage in screening for microbes of potential industrial application is their isolation (Stanbury *et al.*, 1995), and subsequent selection and maintenance of pure, stable cultures. Unless expertly handed, macrofungi, like other microbes, are vulnerable to contamination, spontaneous mutation, deterioration, and death (McNeil and Harvey, 1990). In industrial terms, reisolating or replacing cultures represents additional costs and loss of time. Choosing a culture is often a compromise between its productivity and economic constraints of the process. Its stability, required growth conditions, and the ease of product recovery, together with toxicity, are also important criteria. A wide variety of isolation methods exist to recover fungi from their natural environment, but most depend on some form of enrichment technique (see Stanbury *et al.*, 1995 for a description). In terms of their preservation and maintenance, it is advisable to prepare frozen stock cultures as soon as possible after their isolation (Walser *et al.*, 2001). Plate cultures are prepared and sliced into small squares, which are transferred into 1 ml of sterilized 15% glycerol in cryotubes for freezing at $-80\,^{\circ}C$. For reculturing, the whole vial is defrosted and its contents emptied onto fresh medium, discarding glycerol separately. Working cultures are produced from these frozen stocks, and maintained for some months by regular subculturing before restarting from another frozen stock. The length of the culturing period depends on the strain stability (Walser *et al.*, 2001).

C. Effects of process variables on growth and product formation

Growing mycelium in submerged culture on synthetic or complex media, or on waste substrates is similar to growing ''lower fungi'' or yeasts in submerged culture, but without any sporulation step during inoculum preparation, since generally vegetative inocula are employed (Eyal, 1991). Factors influencing the performance of a bioprocess can be categorized into physical, chemical, or biological (Fig. 2.6). The physical and chemical factors define the environment of the biocatalyst, while the biological factors describe its behavior. The optimal fermentation conditions depend on the nature of the product desired, that is, whether it is biomass or a metabolite (polysaccharides, enzymes, and so on) and the strain of fungus used. Moreover, mycelial growth rate achieved and fungal morphology depend on the culture conditions and the culture media chosen, as discussed later.

Most publications on SLF of macrofungi fail to provide any information on the physiological state of the cells. Furthermore, macrofungi are complex organisms, and relatively little information is available on their

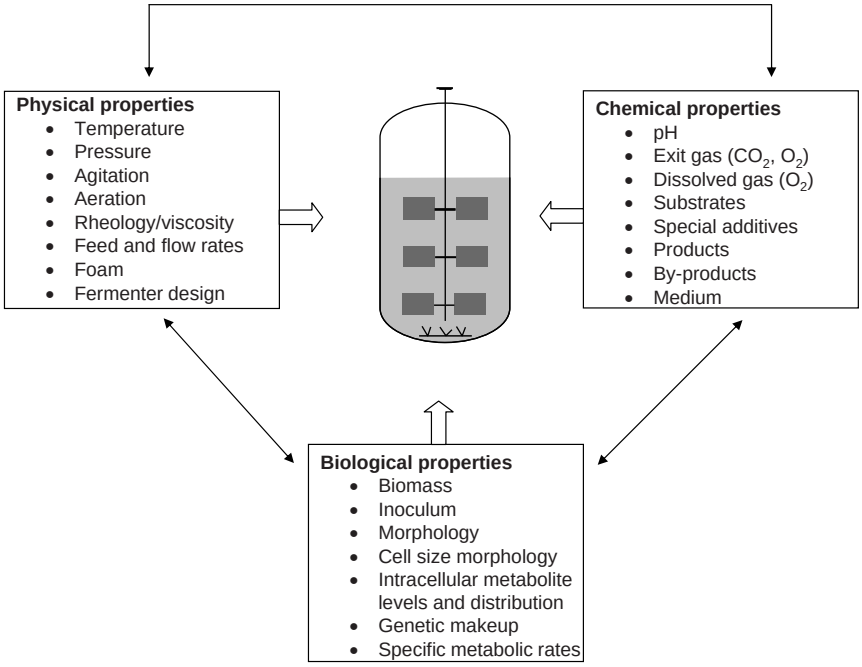

FIGURE 2.6 Factors that influence the performance of a bioprocess and the complexity of interactions between them. The factors are grouped under the three system properties, namely, physical, chemical, and biological. (Adapted from Vaidyanathan et al., 1999).

genetics and enzymatic activities compared with the ''lower fungi'' commonly used in the biotechnology industry. Here we discuss the relevant material published in the last 10 years, to review current knowledge of macrofungi fermentations and to highlight gaps in our knowledge.

1. Physical factors

In general, physical factors like process temperature, agitation rate, and fluid rheology influence the macroenvironment of the biocatalyst, contributing to its morphological and physiological behavior, and in turn affecting the performance of the bioprocess (Vaidyanathan *et al.*, 1999). In this section we discuss how some physical factors affect macrofungal fermentations.

a. Temperature In SLF of macrofungi, temperature is an easy parameter to control. It affects other culture variables such as the growth rate, dissolved oxygen tension (DOT), rate of medium evaporation, pellet formation, and product formation (Papagianni, 2004). Growth of such

mycelial cultures of macrofungi has been examined in SLF at temperatures between 25 °C and 36 °C (Eyal, 1991; Litchfield, 1979).

Increasing temperature generally results in higher metabolic rates, but decreases the solubility of dissolved oxygen in the medium. Small variations in temperature can greatly reduce the productivity. For example, lipase production by *Antrodia cinnamomea* (Lin and Yang, 2006) was influenced by temperature, while mycelial growth was not. Lee *et al.* (2007) reported different optimum temperatures for the production of cell biomass, and endopolysaccharide (10 °C) and exopolysaccharide (EPS) (25 °C) for *Ganoderma applanatum* submerged cultivation. Both cell biomass and endopolysaccharide decreased as the culture temperature increased, suggesting that mycelia intracellularly accumulated polysaccharide at low temperatures. Similar results for EPS production have been published for *Agrocybe cylindracea* (Kim *et al.*, 2005) and *Grifola frondosa* (Lee *et al.*, 2004).

However, most of the published studies are descriptive rather than explanatory (Kim *et al.*, 2005; Lee *et al.*, 2004; Park *et al.*, 2002; Xu *et al.*, 2003; Yang and Liau, 1998a). More time should be spent on investigating the basic physiologies of these fungi to provide the fundamental information about the behavior of macrofungi cultured in fermenter vessels, which is still seriously lacking. Consequently, in the absence of such knowledge on process physiology, in many cases, we are reduced to extrapolate data from experiments on ''lower fungi,'' the relevance of which may be questionable. More systematic studies on optimizing performance of higher fungi in SLF, coupled with the design of experiment (DOE) approach, may assist in untangling the complex web of interacting variables.

b. Agitation Agitation rate and impeller speed play an important role in determining the growth rate of macrofungi, by influencing mixing, and mass and heat transfer. In bioreactors, there are often marked mass transfer gradients throughout the vessel in substrate, products/byproduct, and oxygen levels, affecting both mycelial growth and product formation. More especially, with the continuously stirred-tank reactor (CSTR), for filamentous organisms, mixing and oxygen transfer may only be adequate around the impeller, with essentially stagnant zones elsewhere, subjecting the bulk of the fermentation broth to limiting supplies of oxygen and poor mixing conditions (see Section II.C.1.c). Agitation also maintains a concentration gradient between the interior and the exterior of the cells, which, through increased diffusion rates, helps to establish a satisfactory supply of nutrients to the cells, and facilitates removal of waste gases and other byproducts of catabolism from the microenvironment of the cells (Oh *et al.*, 2007). Also, in aerobic processes, appropriate mixing is required to ensure sufficient oxygen

supply to the cells throughout the vessel, which is important in these aerobic fungi for promoting high biomass concentrations.

However, the shear forces created by agitation can affect the mycelium in several ways. They may damage cell structure, lead to morphological changes, and cause variations in growth rate and product formation (Papagianni, 2004). Therefore, the optimum agitation rate represents a balance between achieving adequate oxygen transfer into the medium and shear stress, both of which increase with increasing agitation rate (Wagner *et al.*, 2003). Furthermore, the rheological properties of the fermentation broth depend on mycelial biomass (and biopolymer) concentrations, together with culture morphology, under different agitation conditions (McNeil *et al.*, 1986). Although the influence of agitation rate on the growth of filamentous microfungi has received considerable attention with the ''lower fungi'' (Gibbs *et al.*, 2000; Papagianni, 2004), fewer studies discuss its importance in submerged cultures of macrofungi.

Oh *et al.* (2007) studied the effect of agitation rate on polysaccharide–peptide complex formation in submerged mycelial cultures of *Cordyceps sphecocephala* J-201 in a 5-liter bioreactor with a six-bladed disc turbine impeller. Increasing agitation speed from 50 to 300 rpm led to an improvement in utilization of all sugars tested. Highest mycelial biomass (28.2 g/liter) and EPS levels (2.50 g/liter) were obtained after day 10 and 11 with an agitation speed of 300 and 150 rpm, respectively. Pellets became smooth and compact with a dense core region as agitation rate increased. The most favorable morphology for EPS production corresponded to a looser pelleted form achieved at 150 rpm. The apparent viscosities of the broth increased rapidly towards the end of fermentation at both increased aeration and agitation conditions, to high values (3000–4000 mPas). This increase arose in the later stages of fermentation from production of high levels of mycelial biomass rather than EPS production. These trends differ from the findings of studies using other entomopathogenic fungi (Kim *et al.*, 2003; Xu *et al.*, 2006). Furthermore, EPS production was favored at mild agitation rates (166 rpm) with *Gr. frondosa* grown in a 5 liter bioreactor (Lee *et al.*, 2004), and increased agitation rates gave lower EPS yields. The authors proposed that this resulted from the shear inactivation of key enzymes involved in polysaccharide synthesis, or to fragmentation of the mycelia, or both. Although, some morphological data were given, and morphological changes noted, it was not possible to elucidate the precise cause of such morphological shifts, since other operational conditions (pH, aeration, and agitation rate) also varied.

Decolorization of recalcitrant dyes by *Ganoderma* WR-1 was better in agitated cultures than in stationary cultures (Revankar and Lele, 2007), and the organism formed uniform pellets and filamentous mats at the medium surface in the agitated and static cultures respectively. The authors attributed increased dye decolorization to the physiological

state of the fungus as pellets and increased mass transfer between the cells and the medium. They also observed dye adsorption onto fungal mycelium only in static cultures, suggesting that in agitated cultures the isolate could degrade it by extracellular activity of oxidative enzymes, such as laccase (see later). Unfortunately, this conclusion is not supported by quantitative evidence. Yang and Liau (1998b) investigated the effects of changing impeller speed on mycelial growth and EPS formation in shake flask cultures of *Ganoderma lucidum* between 50 and 250 rpm. The maximum mycelial concentration was observed at 100 rpm, yet highest yields of EPS were obtained at 150 rpm. Both biomass and EPS yields decreased above 100 and 150 rpm, respectively. It was proposed that a higher speed favored EPS release to the medium, instead of it sticking to the mycelial pellet, stimulating the secretion of further EPS. The same authors verified that at an agitation speed of 400 rpm in a bioreactor, rapid formation of polysaccharide occurred in the early stages of the fermentation, but its concentration then fell rapidly after 3 days, which was attributed to harmful effects on mycelial growth because of shear at high agitation speeds. However, the mechanisms by which this might occur were not discussed. The sizes of the pellets formed were also affected by impeller speed: at low speed (50 rpm), larger pellets formed (diam. 40 mm), while at higher speed (>100 rpm), pellet diameter was less than 5 mm. Later studies, with *G. lucidum* in a 10-liter STR mixed by three Rushton turbines, confirmed that the mycelia of this organism were shear sensitive (Berovic *et al.*, 2003). Agitation speeds over of 300 min^{-1}, led to shear forces that markedly damaged mycelial agglomerates and their peripheral hyphal growth zones, as the number of cut filaments (ratio of the number of cut hyphae to the total number of hyphae) increased drastically.

Rau and collaborators have published several papers on production of β-glucans by filamentous fungi in submerged cultivation, in particular, schizophyllan produced by *Sch. commune* (Gura and Rau, 1993; Rau and Brandt, 1994; Rau *et al.*, 1992; Rau, 2004). For example, Gura and Rau (1993) showed that the agitator design used in their bioreactor affected glucan production. Power requirements, glucan productivity, and quality (expressed as specific shear viscosity) of the aqueous β-glucan solutions were affected by different agitator designs (Rushton turbine, helical ribbon, fundaspin, and three fan impellers of different sizes). A maximum rate of b-1,3-D-glucan formation of 8.8 g/liter/day was achieved with a fan impeller (with a impeller/vessel diameter ratio of 0.64), and increasing impeller speed from 100 to 200 rpm improved oxygen supply at the point of oxygen limitation. Rau (2004) concluded that shear stress created by the agitator reduced pellet growth and enhanced release of the β-glucan from the cell wall. However, too high a shear stress damaged the hyphae, and the β-glucan itself, impeding cell separation during

subsequent downstream processing. Therefore, for these cultures, agitation rate has to be a compromise between achieving effective mixing and mass transfer in a highly viscous, pseudoplastic suspension, and preventing shear damage to the fungus and β-glucan.

Some studies have sought to protect fermenter cultures of macrofungi from the detrimental effects of vigorous agitation by applying selective additives to the medium. Generally, these have been viscosifying agents. Thus, Shu and Wen (2003) enhanced shear protection and increased production of EPS by *Agaricus blazei*, by adding xanthan to submerged cultures. The use of supplemental water-soluble polymers, for example, xanthan, or serum, and pleuronics (successfully used in animal cell cultures) may create a shear-protective environment for polysaccharide biosynthesis. However, these could have a negative effect on O_2 transfer, creating oxygen-limiting conditions, which may only be overcome partially by increasing agitation rates.

To achieve optimal growth of any fungus, it is desirable to maximize the number of growing points, which are their hyphal tips. This can be achieved, at least in principle, by breaking up pellets or large mycelial aggregates, generating smaller pellets or dispersed mycelial forms, which implies using high impeller speeds, subsequently increasing the oxygen transfer rate (OTR). Vigorous agitation has been shown in ''lower fungi,'' like *Aspergillus*, to prevent or limit pellet formation, or to produce smaller and more compact pellets, and to increase the density, thickness, and branching of hyphae filaments (Gibbs *et al.*, 2000). Mechanical forces act by shearing off productive hyphae, thus restricting pellet surface extension. By contrast, when agitation rates are low, oxygen and mass transfer rates are also low, resulting in lowered growth rates and often, in the case of macrofungi, larger pellets, which can lead to substrate difusional limitation. Lower speeds are generally associated with optimal EPS formation, as exemplified previously. The range of agitation rates used in other filamentous fungi processes using *Aspergillus* and *Penicillium* strains are generally much higher than those used with macrofungi (usually between 100 and 400 rpm). With *Aspergillus niger*, higher speeds (1000 rpm) led to lower citric acid yields, although growth rates were high (Papagianni, 2004), when compared with those at lower speeds (450 rpm). Thus, it may be that macrofungi are more susceptible to shear stresses caused by mechanical forces than are other filamentous fungi, a view which is supported by some of the previously discussed data, where ''low shear'' impellers led to higher productivities of EPSs (Gura and Rau, 1993). Of course, low agitation implies poor OTRs, but this may not be such a problem in macrofungi fermenter cultures whose oxygen requirements may be lower than those of microfungi. For example, gassing rates of 1 vvm are commonly used in lab cultures of macrofungi, whereas gassing rates can be much higher with ''lower fungi.''

However, even though EPS production is strongly coupled to growth, the optimum requirements for maximum productivity of polysaccharide are not always the same as those for biomass production. Thus, Rau and Brandt (1994) demonstrated that controlling the oxygen requirements just above oxygen limitation, achieved by variation of the impeller speed, resulted in maximum glucan production, corresponding to minimal biomass and ethanol production. Therefore, for EPS production by macrofungi, mixing conditions should be such that short mixing times, high mass transfer, polysaccharide release from the cell wall, and moderate shear stress on the fungus and the polysaccharide are allowed. Nevertheless, for biomass production, these organisms can be grown at much higher agitation speeds.

Given the diverse nature of macrofungi, such variations in their behavior in response to agitation speeds are hardly surprising, but they make generalization difficult. It is worth critically appraising these reported differences, as this may well lead to questioning of the conclusions reached. First, much of the literature fails to discriminate between the influence of shear forces and that of oxygen supply. These problems can be readily avoided by ensuring the O_2 supply rate is kept constant while stirrer speed is varied, which is achievable with modern fermenter control systems. Second, morphology is rarely measured, but image analysis systems, which quantitatively characterize fungal morphology, would allow relationships between morphology and product formation to be revealed. Third, enzymatic studies, especially quantification of wall-associated enzymes (WAEs) in studies of this type are scarce, and more work is needed to assess changes in the physiological states of the organism under these varying conditions. Moreover, as highlighted by Gibbs *et al.* (2000), the fermenter design, as well as the impact of bioreactor hydrodynamics on hyphal breakage, should be considered (discussed in Section II.C.1.d). Shear stress should be measured not only based on the agitation rates, but also taking into account the impeller geometry (expressed as ''tip speed''), as in the work of Gehrig *et al.* (1998). Finally, a deeper approach to understanding the rheology of the fermentation broth should not be ignored.

Thus, there is both a need, and now a capability with current analytical capabilities, to carry out systematic research linking physiology to fermenter environment.

c. Aeration Aeration is a critical parameter since it is one of the primary energy inputs to the fermentation, and together with agitation, normally satisfies oxygen demand of the cultured cells. Dissolved oxygen concentrations change about 10 times faster than the cell mass and other substrate concentrations, making it the most important physiological variable to control and optimize in aerobic fermentations (Gomes and Menawat, 2000). Oxygen requirements for product synthesis or organism

growth depend on the bioenergetics of the metabolic steps leading to synthesis of the product or cells. Oxygen regulates key biosynthetic enzymes, and thus may directly determine final product yields. Oxygen supply affects cell growth, morphology, nutrient uptake rates, and metabolite biosynthesis rates (Gomes and Menawat, 2000; Tang and Zhong, 2003). Many studies targeted at determining effects of aeration on biomass and product formation in fungi neither control nor monitor dissolved oxygen in the medium, nor perform any ''off gas'' analyses or measurement. They are instead either performed in shake flasks or under different aeration rates in bioreactors, where the aeration rate is usually kept constant by increasing the agitation rate (Emelyanova, 2005; Yang and Liau, 1998b). Therefore, in these studies it is impossible to evaluate any effects of aeration quantitatively, especially independently of agitation rates.

However, some quantitative data on the effects of aeration conditions on mycelial growth and production of metabolites in macrofungi fermentations have been published. These include the works of Oh *et al.* (2007), Park *et al.* (2002), Rau and Brandt (1994), Rau *et al.* (1992), Shu and Wen (2003), Tang and Zhong (2002), and Tang and Zhong (2003). For example, Kim *et al.* (2002) reported that high aeration rates (2.0 vvm) were suitable strategies for both growth and EPS production in liquid cultures of *Pa. sinclairi*. Tang and Zhong (2003) also investigated the role of oxygen in the production of mycelial biomass of *G. lucidum* and its metabolites (EPS, intracellular polysaccharides (IPS), and ganoderic acid (GA)). Initial volumetric oxygen transfer coefficient values (K_La) markedly affect cell growth, morphology, and metabolite production. The K_La is normally used as a measure of the capacity of the fermentation system to transfer oxygen from the gaseous to liquid phase (Stanbury *et al.*, 1995), K_La is the average of the difference between equilibrium oxygen dissolved concentration C^* (kg/m^3) and dissolved oxygen concentration C (kg/m^3) and is proportional to the OTR in a fermentation broth. For example, in antibiotic fermentations with microfungi, K_La values between 70 and 250 h^{-1} should be provided for high biomass concentrations (Znidaršic and Pavko, 2001). In the study of Tang and Zhong (2003), maximal dry cell weight (15.6 g/liter) was obtained after 12 days with an initial K_La of 78.2 h^{-1} at 0.5 vvm. An increased initial K_La (over a range of 16.4–96.0 h^{-1}) led to larger mycelial aggregates and a higher production of EPS. Again, no systematic morphological characterization of the fungus was carried out. Interestingly, although cell growth of *G. lucidum* and production of both IPS and GA fell markedly when the DOT was controlled at (10% saturation; EPS production increased, suggesting that each metabolic activity had different oxygen requirements. Cui *et al.* (2006) reported that pellet size, hairy length of pellets, and free filamentous mycelial fractions in the total biomass of *Gr. frondosa* were independent

of DOT level provided that the DOT was neither too low nor too high. They also pointed out that pellet size was hardly affected by DOT, yet the fraction of free mycelia decreased slightly with an increase in DOT. Oh *et al.* (2007) observed a considerable variation in mycelial morphology under different aeration conditions in their bioreactor culture of *Co. sphecocephala*. Looser mycelial pellets developed, and their size and hairiness increased as aeration rate increased from 0.5 to 2.0 vvm, resulting in enhanced EPS production. Increases in DOT should lead to higher OTRs and oxygen uptake rates (OURs) by these cells. Furthermore, oxygen supply to the pellet interior will decrease as a function of the pellet diameter, which can be one of the limiting factors in the growth of macrofungi. To determine if such DO limitations exist, the critical diameter of the pellets can be calculated according to the following equation (Tang and Zhong, 2003):

$$d_{\text{crit}} = \sqrt{\frac{24 \times C_{O_2} \times D_{\text{eff}}}{R_{O_2}}}$$

d_{crit} is the critical pellet diameter at which the internal oxygen limitation will occur, C_{O_2} is the DOT in the medium, D_{eff} is the effective diffusion coefficient of oxygen in mycelial pellets, and R_{O2} is the oxygen consumption rate per pellet volume. D_{eff} is equal to the product of molecular diffusion coefficient and the pellet porosity (1.9×10^{-9} m^2/s, Biryukov and Tarasova, 1974). R_{O_2} is equal to the product of specific oxygen uptake rate (SOUR) and the density of the mycelia pellets.

Few studies have used oxygen enrichment in the submerged cultivation of macrofungi (Hsieh *et al.*, 2006; Shih *et al.*, 2006; Shu and Wen, 2003), suggesting that either these organisms do not require extra oxygen during their submerged growth (depending of course, on the aim of the process), or that their respiratory requirements have not been carefully investigated. In which case, more time should be spent on monitoring O_2 profiles and consumption rates, instead of opting for an empirical approach. The former certainly fits with our basic knowledge of these relatively slow growing cultures, and logically, for a slow growing aerobe, oxygen demand will be concurrently low. However, this conclusion is based on limited data and should not deter us from systematic investigations of the metabolic limits of these important microbes.

Two studies have focused on the effect of enriched oxygen supply on the production of mycelial biomass and EPS by *Gr. frondosa* (Hsieh *et al.*, 2006; Shih *et al.*, 2006; Shu and Wen, 2003) and *An. cinnamomea* (Hsieh *et al.*, 2006; Shih *et al.*, 2006; Shu and Wen, 2003) in bioreactors. Both cultures were aerated at a rate of 1 vvm with inlet air containing different percentages of oxygen. In the *An. cinnamomea* culture (Hsieh *et al.*, 2006; Shih *et al.*, 2006; Shu and Wen, 2003), high O_2 supply

(30% saturation) favored cell growth and polysaccharide production, but inhibited tripernoid production. Shu and Wen (2003) reported that regardless of impeller tip speed, high O_2 supply rates favored cell growth, while low rates favored the biosynthesis of EPS. With *Gr. frondosa*, the highest oxygen concentration used (40%) inhibited both cell growth and polysaccharide production, but a higher rate of glucose consumption was recorded. Also, by monitoring mycelial morphology with scanning electron micrographs, clear ''wrinkles'' on mycelial surfaces appeared when 40% O_2 was used from the beginning of the experiment. Olive oil addition to the medium reduced these ''wrinkles'' appearing, and made the cells age slower.

Controlling the aeration rate effectively contributes to increasing the overall productivity of submerged cultivation of macrofungi. Furthermore, it is essential to develop strategies which control dissolved oxygen levels precisely, especially in highly viscous, larger-scale fermentations. At the intracellular level, monitoring the rate of respiration (NADH/NAD levels) with cell age and comparing these with the activity of key enzymes involved in carbohydrate metabolism is worth pursuing. Rau and Brandt (1994) developed an oxygen-controlled batch fermentation of *Sch. commune* where the SOUR was used as the master variable, and the impeller speed as a correction variable. By maintaining optimal morphology (mycelial growth), and by modeling biomass based on carbon and oxygen balances, both productivity (4.3 $kg/m^3/day$) and yields of glucan (13 kg/m^3) could be markedly increased more compared to those in an uncontrolled batch culture (productivity 2.4 $kg/m^3/day$, yield <10 kg/m^3). The potential in adopting a more systematic approach to higher fungal fermentation processes becomes very clear from this example.

CO_2 production occurs as an inevitable consequence of cell respiration in aerobic fermentations. Online measurement of the CO_2 evolution rate (CER) is valuable as it directly correlates with cell growth, and can give rapid information on the physiological state of the cells in the fermenter. CO_2 production in the broth may influence the morphology of fungi, and be an essential prerequisite for both organism growth and product formation. However, it can also inhibit fungal growth at least in microfungi. For example, in the filamentous fungus *Penicillium chrysogenum*, elevated CO_2 caused lower broth viscosity, and hence better oxygen transfer by stimulating chitin synthesis in the cell wall, but such level also inhibited penicillin production (Znidaršic and Pavko, 2001). Thus, any effects of CO_2 might have with macrofungi should be investigated and monitoring the CERs over the culture period may suggest how these might be used for process control. A simple inexpensive gas analyzer that measures the off gases of a bioreactor makes it possible to quantify both O_2 and CO_2, and reflects the physiological state of the culture. This step should be

routinely adopted in all SLF studies on macrofungi to fill in the gaps in our knowledge of the quantitative physiology of these organisms.

d. Fermenter design In most CSTR fermentations of macrofungi, standard radial flow Rushton impellers are the most commonly used mixing system, probably because they are those routinely supplied by fermenter manufacturers. Most fermenters are optimized for high OTRs and rapid growth of aerobic microbes. The Rushtons provide some flexibility for a range of microbial cell types, and can be used with some animal cells too. However, Rushton turbines impart high shear stresses on the medium, and their use, particularly at high speeds, may have a detrimental effect on product yield with shear-sensitive cells. Unfortunately, little work, if any, has been carried out to establish whether such effects are seen with macrofungi. Most studies have used shake flask culture systems and low shaking speeds of 50–250 rpm. Several alternative impeller designs or configurations are known to be more effective in achieving bulk liquid mixing in the viscous fermentation fluids distinctive of filamentous fungi. Therefore, it would be valuable to explore such alternative mixing systems in SLF of macrofungi (e.g., axial flow impellers offer superior mixing, especially in larger-scale cultures, with a view to reducing shear damage). Only a single investigation has been reported where EPS production by *Sch. commune* was compared in vessels equipped with axial flow, helical ribbon impellers, and Rushton turbines (Rau *et al.*, 1992), and the agitation system shown to influence yields. More studies are clearly needed.

The use of airlift fermenters seems a promising approach to increase mycelial biomass yields and polysaccharide production in macrofungi at reduced mechanical shear. Airlift fermenters present several advantages over CSTRs. They do not require mechanical agitation, and therefore, their shearing effects are considerably lower. Subsequently, the energy costs involved are less, and the risks of contamination reduced, since no complex mechanical seals are required on the impeller shaft (Gibbs *et al.*, 2000). By contrast, airlift fermenters are not as effective as CSTRs at O_2 mass transfer. Studies have sought to compare yields in airlift and stirred-tank fermenters with macrofungi (Table 2.2). Nevertheless, again they appear to be rather superficial and descriptive. Biomass yields and EPS concentrations were both lower in an airlift than in a CSTR with *Gr. frondosa* (Lee *et al.*, 2004). Morphological features were also different, with compact pellet formation in the airlift fermenter compared with feather-like mycelial clumps in the stirred-tank system. However, no quantitative (image analysis) results were presented. A similar study (Cho *et al.*, 2006), using *Tremella fuciformis*, gave different results, and all kinetic parameters in the airlift fermenter, including specific productivities and yield coefficients of both cell mass and EPS, were higher than those achieved in the CSTRs. The most favorable morphological form for

TABLE 2.2 Comparing metabolite yields in "higher fungi" grown in STR with rushton turbines to those obtained in fermenters with different configuration (adapted from Gibbs *et al.* (2000))

Fungus	Configuration	Metabolite	Influence on yield	Reference
Scl. glucanicum	Airlift with external loop	Scleroglucan	Comparable to STR	Wang and McNeil (1995)
Sch. commune	Draft tube/ propeller system	β-Glucan	Increase	Rau *et al.* (1992)
	Fan impeller		Increase	
	Helical ribbon		Decrease	
	Fundaspin		Decrease	
	Intermig		Increase	
Tre. fuciformis	Airlift	EPS	Increase	Cho *et al.* (2006)
Gr. frondosa	Airlift	EPS	Decrease	Lee *et al.* (2004)
G. lucidum	Airlift	EPS	–	Lee *et al.* (1999)
Ga. applanatum	Airlift with external loop	EPS IPS	–	Lee *et al.* (2007)

EPS production (elongated yeast-like form) by *Tre. fuciformis* was maintained longer in the airlift fermenter. These results suggest that the performance of a culture in a bioreactor is markedly affected by the morphology of the cells, which may reflect the imposed shear. Adopting novel fermenter configurations may be also rewarding. For example, Gibbs and Seviour (1998) improved mixing and oxygen mass transfer during polysaccharide production by inserting impellers in the draught tube of an airlift fermenter.

e. Foaming Foam formation is undesirable, since it interferes with monitoring and control of fermentation conditions and can complicate product recovery. With the complex media often used, foaming can be a substantial problem. Conventional chemical antifoam agents are sometimes added, especially in large-scale cultivation of macrofungi, but they must be used sparingly and with care, as they can markedly decrease OTRs, with the attendant effects on cell growth (Wagner *et al.*, 2003).

f. Culture time Harvest time in a submerged culture often determines the final quality of the product. For example, in polysaccharide production, production of degradative enzymes, such as β-glucanases, is linked to the culture age (via carbon exhaustion), and therefore, their action may determine both the quality and yield of the polysaccharide (Shu and Wen, 2003). The former is usually expressed in terms of molecular weight, with high molecular weights having the more desirable biological properties (>1000 kDa). Shu *et al.* (2003) described a method for monitoring the biological activity of broth polysaccharides in a submerged culture of *A. blazei*. They showed a direct correlation between the release of TNF-α (a yielding tumor necrosis factor-α) and polysaccharide molecular weight, using an *in vitro* mouse macrophage RAW 264.7 cell line. In the submerged cultures of *Ga. applanatum* (Lee *et al.*, 2007), the longer the cultivation time, the higher the molecular weight of EPS obtained, resulting in a viscous, almost gel-like broth, where cells adhered and coagulated to form large clumps. Also, more EPS was produced during the stationary phase, while more IPS accumulated in cells in the decline stage. Equally extracellular lipase from *An. cinnamomea* displayed maximum activity in samples harvested during late logarithmic phase (Lin and Ko, 2005). Consequently, culture age may have a profound effect on the product of interest, and therefore, should be taken into consideration as an optimization parameter.

2. Chemical factors

a. pH of the medium In submerged cultivation of all organisms, including the macrofungi, initial pH of the medium may affect cell membrane function, cell morphology and structure, solubility of salts, ionic state of the substrates, uptake rates of substrates, the production of bicarbonate from dissolved CO_2, and biomass and metabolite formation (Fang and Zhong, 2002). In turn, initial pH is determined by the composition of the medium. Thus, in a fermentation in which pH is not automatically controlled, pH drifts will probably occur during the growth of the fungus from nutrient use or waste-product excretion; in turn, influencing fungal growth and product formation (Papagianni, 2004). In general, macrofungi seem able to grow over a wide range of pH (Worgan, 1968), and it is generally desirable to operate at a pH of 5.0 or below to minimize risks of potential bacterial contamination, particularly when fungi are grown on wastes under nonaseptic conditions. Many macrofungi have low pH growth optima (pH 4.0), but higher pH optima for EPS production (pH 6.0) as suggested by the data of Kim *et al.* (2005) and Jonathan and Fasidi (2003). However, these statements do not apply to all systems (Fang and Zhong, 2002; Kim *et al.*, 2003; Xu and Yun, 2003). The optimum pH will depend on several factors, including the strain of the organism used (Xu and Yun, 2003), as discussed later.

The pH shifts are usually caused by excretion of waste products, including organic acids (Berovic *et al.*, 2003; Fang and Zhong, 2002; Jonathan and Fasidi, 2003; Kim *et al.*, 2005; Xiao *et al.*, 2006), into the medium. However, very few reports identify the compounds involved. When complex media (e.g., yeast extract and peptone) are used, the pH increase is from secretion of proteases that degrade the complex nitrogen sources (Delgado-Jarana *et al.*, 2002). The pH rises noted may result either from culture autolysis or from deamination of amino acids used as carbon and energy sources.

Glucose consumption rates can be strongly influenced by pH of the medium (Kurosumi *et al.*, 2006). In *Sparassis crispa* mycelial cultures in shake flasks, glucose was most rapidly consumed at pH 5.0 over an initial pH range of 4.0–9.0, and no growth and glucose consumption occurred at pH 9.0. Similar trends from *An. cinnamomea* (Lin and Yang, 2006; Lin *et al.*, 2006; Yang *et al.*, 2003) and *G. lucidum* (Fang and Zhong, 2002) have also been published.

Kim *et al.* (2005) studied the effect of controlled pH on growth, EPS production, morphology, rheology, and substrate consumption rates by *Cordyceps militaris* C738 in a 5-liter stirred-tank fermenter. They showed that in pH-controlled cultures (6.0) EPS production was enhanced (7.3 g/liter) compared to uncontrolled conditions (2.3 g/liter). On the other hand, higher mycelial biomass was achieved without pH control (25.7 g/liter compared to 12.7 g/liter). However, the growth period for each of the cultures described above was different, making comparisons difficult. Although Park *et al.* (2001) obtained similar results to these, it is again difficult to draw meaningful comparisons between the data, since they were obtained from experiments performed at different scales. For the submerged culture of *G. lucidum* (Yang and Liau, 1998b), pH was maintained at 4.0 in a fermenter culture, but this had a negative effect on EPS production, and production fell by 30% after 7 days. A similar result was reported by Kim *et al.* (2002). Yang and Liau (1998a) also verified that optimal pH depended on medium composition, and for *G. lucidum* was lower (4.0) in a chemically defined medium than in a complex medium (5.0, glucose–malt extract medium). Many fungi can grow at a range of initial pH values because they can regulate the medium acidity to levels supporting their growth (Emelyanova, 2005). In general, when ammonium salts are used as the nitrogen source, the pH decreases as the result of assimilation of the ammonium ion and proton extrusion, and the effects of acidic anions in the medium such as chlorides, sulfate, or phosphate. The limits of this pH decrease depend upon the buffering action of both medium and mycelium (Yang and Liau, 1998a).

Lee *et al.* (1999) suggested a bistage control technique to enhance EPS production in an airlift fermenter by *G. lucidum*, where the pH value for optimal mycelial growth (3.0) was changed to a value more favorable to

EPS production (6.0). They also claimed that the different morphologies produced at different initial pH values were critical in affecting biomass accumulation. In uncontrolled pH conditions (initial pH 6.0), morphology changed from a pellet-like form to a filamentous form, data contrary to those of Fang and Zhong (2002), where the pellet profile of *G. lucidum* was similar regardless of initial pH values. Fang and Zhong (2002) also suggested that lowering initial pH from 6.5 to 3.5 gradually led to higher production of EPS and higher specific production of intracellular polysaccharide, but maximum biomass and ganoderic acid formation occurred at 6.5. Higher respiratory activity was also observed at a high initial pH, where better cell growth occurred. However, the reduction in polysaccharide synthesis at low pH in the ''lower fungi'' *Sclerotium glucanicum* was considered to be from an increase in the amount of energy source (carbon source) required for maintenance purposes (Wang and McNeil, 1995). This would cause a shift in carbon flux to cell production from product formation, which concurs with the findings of Lee *et al.* (1999).

b. Medium composition Medium optimization is often the key for successfully growing macrofungal mycelium. Synthetic chemically defined media, complex media, and waste substrates have all been used. It is important to bear in mind that each component of the culture system has a multiplicity of interconnected effects, and so there can be no generalized ''ideal'' medium. Also, some media support the growth of some strains better than others do, which means that each needs to be studied individually. The medium chosen has to promote both growth and product formation (Papagianni, 2004).

Some general important conclusions can be stated for growing macrofungal mycelia in submerged culture (adapted from Eyal, 1991). Although many of these are relevant to the growth of other fungal species, it is important to bear them in mind when trying to grow the macrofungi:

a. High concentrations of carbohydrates are usually needed in order to achieve a high yield of mycelium (dry weight).
b. The carbon:nitrogen (C:N) ratio is important, since it influences the yield and efficiency of the production of mycelium and metabolites (Hsieh and Yang, 2004; Lee *et al.*, 2007; Park *et al.*, 2001; Rogalski *et al.*, 2006). The C:N ratio can influence the protein and lipid content of the mycelium.
c. If wastes sources are used, supplements of nitrogen or minerals are often required (Hsieh and Yang, 2004; Lee *et al.*, 2003).
d. Biomass concentrations are generally lower with synthetic defined media than with complex media (Fan *et al.*, 2007; Lin and Yang, 2006; Xiao *et al.*, 2006).

e. Medium composition affects the secretion of metabolites, like extracellular enzymes (Lin and Yang, 2006; Revankar and Lele, 2007; Rogalski *et al.*, 2006; Songulashvili *et al.*, 2007), and polysaccharides (Kim *et al.*, 2005; Kim *et al.*, 2006).
f. The sugar content/composition of any polysaccharides produced depends on the carbon source (Hsieh and Yang, 2005; Kim *et al.*, 2006; Lee *et al.*, 2007).
g. Efficiency of protein production and flavor formation varies with different carbon and nitrogen sources, and also with the time of mycelial harvest (Humfeld and Sugihara, 1949).
h. Protein content of the mycelium can be controlled by the amount and kind of nitrogen supplied in the growth media (Cheung, 1997; Eyal, 1991).
i. Supplementation of mineral salts and inorganic salts may also influence the flavor and amino acid composition (Mukhopadhyay *et al.*, 2005), and promote mycelial growth (Chang *et al.*, 2006; Hwang *et al.*, 2004; Kim *et al.*, 2006; Lin *et al.*, 2006).
j. Addition of nonionic surfactants, fatty acids, oils (Chang *et al.*, 2006; Park *et al.*, 2002), and lipids, such as esters of oleic and linoleic acid, generally stimulates the mycelial growth rate.

c. Carbon source Carbohydrates are major components of the cell cytoskeleton and important nutrients for growth and development of macrofungi. Patterns of utilization of carbon sources vary between fungal species (Hwang *et al.*, 2004), and carbon source and concentration may have complex effects (Kim *et al.*, 2005; Lin *et al.*, 2006). Popular carbon sources include simple sugars like glucose, sucrose, maltose, and complex sources such as molasses, malt syrup, starch, and bagasse. Many authors have studied the effects of different carbon sources and initial sugar concentration on yields of cells and metabolites (Wymelenberg *et al.*, 2006). For EPS production, disaccharides (sucrose, lactose, maltose, and so on) are generally better than monosaccharides (glucose, fructose, galactose, and so on) (Shih *et al.*, 2006), probably reflecting their relative ease of polymerization (Fan *et al.*, 2007). Among carbon sources used with *An. cinnamomea*, Lin *et al.* (2006) found that sucrose resulted in highest biomass and lipase production, while highest lipase activity (26.69 mU/ml) was with glycerol. The reasons for this are unclear. Normally higher concentrations of the carbon source (>35 g/liter) lead to greater production of EPS, implying a high C:N ratio is important, but good mycelial growth does not always seem to ensure a high yield of metabolites (EPS, lipases) (Kim *et al.*, 2005; Lin *et al.*, 2006; Shih *et al.*, 2006; Sone *et al.*, 1985; Xiao *et al.*, 2006). Xiao *et al.* (2006) showed that a medium containing both molasses and glycerol gave higher mycelial growth and EPS production than did a single carbon source for *Cordyceps jiangxiensis*. Arora and

Gill (2001) studied the influence of medium composition on laccase production by white rot fungi, and found mineral salts and malt broth were best. Among eight complex substrates from food industry wastes examined, highest laccase activity (93–97 U/ml) in the submerged cultivation of *G. lucidum* 447 was obtained with wheat bran and soy bran (Songulashvili *et al.*, 2007). Glucose seemed to stimulate lipase production in *An. cinnamomea* (Lin and Ko, 2005; Lin *et al.*, 2006). Fang and Zhong (2002) noted a decrease in the average growth rate of *G. lucidum* as initial glucose concentrations increased above 35 g/liter and attributed this to an increased osmotic pressure of the medium. Lee *et al.* (2007) showed that both mycelial and EPS production by *Ga. applanatum* increased with increasing concentration of glucose. By contrast, the IPS content decreased. However, under different C/N ratios (where only the nitrogen concentration was changed), EPS production was not affected, yet IPS content decreased at C/N ratio above 43. Thus, EPS production seems more affected by carbon source concentrations and IPS content by nitrogen concentrations in the media. Kim *et al.* (2006) reported that the optimum glucose concentration for EPS production ranged between 35 and 70 g/liter.

Mycelia of many macrofungi will grow to differing extents over a range of C:N ratios, provided all other nutritional requirements are met. These ratios range from 5:1 to 25:1; C:N ratios of 10:1 or less will ensure a high mycelial protein content and a C:N ratio in excess of this figure (e.g., 50:1) will favor accumulation of alcohol, acetate-derived secondary metabolites, lipids, or extracellular polysaccharides (Carlile *et al.*, 2001; Eyal, 1991). Park *et al.* (2001) reported a direct relationship between the C:N ratio and both mycelial growth and EPS production with sucrose and corn-steep powder with *Co. militaris* fermentation, and an optimum C:N ratio of 20:1.

Some macrofungi, under carbon limited conditions, release β-glucanases that may degrade any preformed β-glucan, with an associated drop in the apparent viscosity of the medium (Rau, 2004). These changes normally occur towards the end of a batch process. If the aim is to maximize the yield and quality of EPS, these events should be avoided by either applying appropriate feeding strategies, or by careful timing of harvesting.

d. Nitrogen source Nitrogen sources used in fermentations include inorganic (nitrite, nitrate, ammonium salts) and organic sources (casein, peptone, amino acids). Beet or cane molasses, whey powder, soy flour, corn-steep liquor, Pharmamedia, yeast extract can also be used, since they are nitrogen rich. Nitrogen plays an important role in fungal metabolite overproduction, and also may affect fungal morphology in the macrofungi.

Lin *et al.* (2006) reported that inorganic nitrogen sources supported lower mycelial production, but improved yields of EPS in *An. cinnamomea*.

Organic nitrogen sources also increased biomass yields in submerged culture of *G. lucidum* (Fang and Zhong, 2002), suggesting that certain essential amino acids could not be synthesized from inorganic nitrogen sources by *G. lucidum*. Wasser *et al.* (2003) demonstrated that types and concentrations of nitrogen sources strongly influenced cell growth and polysaccharide production in *Tremella mesenterica*, and combined use of yeast extract and corn-steep liquor enhanced accumulation of extracellular polysaccharide. Unfortunately, as with almost all of this work, these studies are descriptive only and no explanation to experimental data are provided to support the data presented. Also, because macrofungi are generally grown in media containing complex nutrients, it is difficult to evaluate the impact of any individual component on the behavior of these organisms.

By contrast, a chemically defined medium was used with *Co. militaris* to investigate the effect of on secondary metabolite production by Mao and Zhong (2006). Their experimental design allowed any ''cause'' and ''effect'' relationships to be evaluated. When various complex nitrogen sources were compared, they could conclude that even though all supported growth, peptone was best for cordycepin biosynthesis. They also suggested that it was from the derived peptone degradation that played such a crucial role in enhancing cordycepin production. Thus, a fed-batch strategy with ammonium sulfate feeding was designed, and scaled up to a bioreactor (3.5 liter). This systematic study is valuable, and illustrates clearly the value of carefully planned and analyzed experimentation in elucidating macrofungal metabolism.

Many macrofungi, particularly white rot fungi (e.g., *Ganoderma* spp.), are efficient ligninolytic organisms, because they synthesize extracellular nonspecific and nonstereoselective enzyme systems composed of laccases, lignin, and manganese peroxidases (Revankar and Lele, 2007), which allows them to degrade complex substrates (dyes, starch, cellulose). Ligninolytic enzyme production appears to be substrate-dependent, but why some complex substrates induce their production and others do not is not yet clear, as the chemical nature of the inducer is not known (Songulashvili *et al.*, 2007). These organisms generally live on substrates that are low in most nutrients except carbon (Carlisle *et al.*, 2001). Nitrogen content is very low in wood. Moreover, some nitrogen is sequestered in lignin and other aromatics, and is only available to fungi able to metabolize these molecules. So, most of these cultures in nature are N-limited. Therefore, by understanding the mechanisms by which these organisms cope with nitrogen limitation, strategies for their submerged cultivation may be developed. In this context, *Sch. commune* has been most widely examined. The main mechanism to support sustained growth in *Sch. commune* under nitrogen-limited conditions is autolysis of older cells by proteases and subsequent translocation of the nitrogen-containing

molecules (mainly, amino acids) to the growing apical tip cells (Guettler *et al.*, 2003), but little is known about signaling and regulation in macrofungi in contrast to the wealth of knowledge about nitrogen-limitation regulation in *Saccharomyces cerevisiae* (Guettler *et al.*, 2003). Guettler *et al.* (2003) successfully collected ESTs (expressed sequence tags) from *Sch. commune*, allowing the transcriptional responses to nitrogen availability to be followed. They concluded that genes encoding products involved in cell-cycling processes and in protein biosynthesis were more frequently active in the nitrogen-limited and nitrogen-replete library respectively. These results are not surprising considering that nitrogen-limited growth is characterized by markedly reduced biomass yields. Yet, *Nematoloma frowardii*, *Phanerochaete chrysosporium*, and *Clitocybula dusenii* all showed higher ligninolytic activity under nitrogen limiting conditions, and not as a result of carbon limitation, while in submerged cultures of *Bjerkandera* spp. and *Phanerochaete flavido-alba*, ligninolytic enzyme production was enhanced in a high nitrogen-containing medium (Rogalski *et al.*, 2006). Therefore, nitrogen concentration in the medium may affect mycelial yield and metabolite production by different mechanisms. Analysis of intracellular amino acid pool contents with different nitrogen substrates/concentrations, might provide some clues on how nitrogen metabolism is regulated in macrofungi.

e. Complex media Complex sources of nutrients (e.g., waste materials) are commonly used in large-scale cultivation of fungi, since they are cheaper than the synthetic media or otherwise require costly disposal. In addition, many strains grow better in complex media, either because unknown growth factors and trace elements are present, or because these carbon or nitrogen sources are broken down slowly, so that concentrations of their metabolizable products always remain low. Several waste materials have been used with macrofungi. They include olive-mill wastewaters (Crognale *et al.*, 2003), orange peel extracts, asparagus butt juice, pear waste juice, and molasses (Singh, 1998). Complex nitrogen sources used include yeast extract, corn-steep liquor, vegetable wastes like cauliflower leaves, and brewery wastes. Cheese whey was used successfully as a substrate for production of *G. lucidum* mycelium (Lee *et al.*, 2003). It contains about half the nutritional value of milk and most nutrients essential for microbial growth are present. Dairy wastes should be viewed as an inexpensive potential source of raw material from which valuable products can be produced, but they have not been exploited fully.

f. Metals and other ions Essential metals and other ions are required for fungal growth, usually being supplied as salts (e.g. magnesium sulfate and potassium phosphate) at low levels (10^{-3} M) (Vahidi, 1996). Trace elements (iron, copper, manganese, zinc, and molybdenum) act as

cofactors for enzymes. Thus, calcium promoted both mycelial growth and polysaccharide production by *G. lucidum* (Chang *et al.*, 2006). External Ca^{+} has a double effect in fungal growth: (1) it can change the cell membrane permeability by controlling the internal Ca^{+} gradient and activities of fungal enzymes involved in cell wall expansion (Chardonnet *et al.*, 1999); (2) it can inhibit biopolymer synthesis via the internal Ca^{+} gradient, affecting protein and neutral sugar content (Papagianni, 2004).

As might be expected, studies of this kind with macrofungi often give rise to contradictions. For example, Lin *et al.* (2006) showed mineral sources had little or no effect on EPS production by *An. cinnamomea*, but adding $CaCl_2$ increased mycelial growth. Sodium is known to inhibit several processes in macrofungi, including respiration and fermentation, and Jonathan and Fasidi (2001) suggested *L. edodes* and *Sch. commune* grew best in Na-free medium. On the other hand, magnesium and potassium are essential for all fungi, since Mg^{+} is a cofactor in many enzymatic reactions, stabilizes the plasma membrane, and its uptake is ATP dependent. K^{+} regulates osmotic potential and the turgor pressure necessary for hyphal tip extension. Some metal ions are toxic to some fungi like *Psathyenella atroumbanata (Pegler)*, *Agaricus campestris*, and *Volvariella volvacea*, which grew only in the absence of cobalt and manganese (Jonathan and Fasidi, 2001). On the other hand, copper and zinc were essential for their optimal growth. Both are involved in enzymatic reactions, but zinc also plays an important role in intermediary metabolism and synthesis of DNA and RNA (Griffin, 1994).

Clearly any requirements for micronutrients depend on the strain and medium used, and it is not always necessary to add trace elements to the medium, especially if complex media are being used.

g. Vitamins Although some studies on the effects of adding vitamins to cultures of macrofungi have been reported, most are once again only descriptive. For example, Jonathan and Fasidi (2001, 2003) observed that vitamins stimulated growth of *Psathyrella atroumbonata*, *Lentinus subnudus*, and *Sch. commune*. They proposed that some (particularly biotin and thiamine) acted as cofactors for enzymes of intermediary metabolism, such as lipid metabolism. Pyridoxine had the greatest influence, and is normally associated with tryptophan synthesis in these fungi. In *An. cinnamomea* (Lin *et al.*, 2006), of the five vitamins (thiamine, riboflavin, ascorbic and nicotinic acid, and biotin) tested, only riboflavin increased mycelial growth, but none made any difference to EPS production. Lin *et al.* (2006) suggested that adding vitamins was not essential for fungal growth in *An. cinnamomea* probably because it could synthesize them on its own.

Since many macrofungi form intimate relationships with plants in nature, the influence of plant hormones on some macrofungi has been examined in submerged cultures. Both stimulatory and inhibitory effects on their growth have been reported. Mukhopadhyay *et al.* (2005), found that 10 ppm levels of gibberellic acid, naphthalene acetic acid, and 2,4-dichlrophenoxyacetic acid enhanced mycelial growth by *L. edodes* and *Sch. commune* in a synthetic medium. Above this level, growth was inhibited (Jonathan and Fasidi, 2001). Similarly, Mukhopadhyay *et al.* (2005) showed all plant hormones stimulated growth and enhanced the protein content of *Pleurotus sajor-caju* in a whey medium. Greatest improvement in biomass yields (28%) occurred with indole-3-acetic acid (IAA), which is often used to promote initiation of root growth in plants. Tomita *et al.* (1984) proposed that this class of plant hormone might also influence fungal cell elongation and differentiation although how this might occur was not discussed.

h. Special additives Fatty acids, plant oils, and surfactants are frequently used as antifoam agents, but may also stimulate growth of macrofungi as well as metabolite production in submerged cultures (Certik *et al.*, 1997; Fukushima *et al.*, 1991; Park *et al.*, 2002; Song *et al.*, 1989). Plant oils have the added advantage of being reasonably cheap, and offer the possibility of simultaneously stimulating growth and suppressing foam, whereas most commercial antifoam agents (silicone based) achieve only the latter. Adding safflower seed oil, markedly increased the mycelial yield in *G. lucidum*, while olive oil gave increased polysaccharide yields (Chang *et al.*, 2006). However, contradictory results were reported by Yang *et al.* (2000), who showed EPS production in this organism was highest with safflower oil, which might reflect differences in cell morphology. Yang *et al.* (2000) and Park *et al.* (2002) proposed that such stimulation arises from incorporation of their fatty acids into the cell membrane, rendering them more permeable thereby facilitating both nutrient assimilation and metabolite excretion. However, they could not demonstrate any clear relationship between the length of the carbon chain of the different fatty acids in oils and the level of unsaturation of the membrane lipids. Park *et al.* (2002) also reported that 2% (v/v) oleic acid and palmitic acid markedly stimulated EPS production of *Co. militaris*, but linoleic acid suppressed both mycelial growth and EPS production. Again, no explanations supported by any experimental data were provided.

i. By-products By-product formation can affect the overall productivity of a fermentation process, as with *Sch. commune*, which forms not only EPS but also ethanol (Rau, 2004). While high pH values appeared to favor oxalic acid and glucan production in these cultures, lack of oxygen repressed the oxalic acid synthesizing enzyme (glycolate oxidase).

Therefore, it may be necessary to consider all factors likely to encourage production of any unwanted by-products during submerged culture, as this represents wastage of the supplied carbon source. The best approach to systematically investigate by-product formation by macrofungi during their submerged culture is from complete mass balances. To limit the study of these organisms in order to quantify only biomass and polysaccharide (or other major product) levels, as is most frequently done, is clearly risky. If substantial by-product formation is occurring, it may be possible to reduce it and redirect substrates to desirable destinations by the now standard techniques of metabolic control or engineering.

3. Biological factors

a. Inoculum It is widely accepted that inoculum concentration and form exerts a major influence on the fungal fermentation profile. In fact, the amount, type (spore vs. vegetative), age, and viability of the inoculum all may affect morphological state of the cells, especially in pellet production and the type of pellets produced (Gibbs *et al.*, 2000). However, few reports on inoculum effects on submerged cultures of macrofungi exist. Unlike other filamentous fungi that readily produce spores which can be used as an inoculum, this is not as practical as with most macrofungi, since sexual spores are only formed once the fruiting body has matured (Wagner *et al.*, 2003). Therefore, mycelium-based inocula must be used, which makes their standardization difficult. Most workers use small pieces of mycelium still attached to the agar on which the fungi were grown, and inoculate these directly into the fermentation broth (especially for shake flasks). On the other hand, submerged seed culture is often used to inoculate a bioreactor. In the former, the mycelium has to adapt from a solid to a liquid environment, ensuring long lag phases and low inoculum densities. In the latter, the nutritional and environmental conditions used to prepare the seed culture should be the same as those in the fermentation to avoid lag phases. Also, for inoculum standardization either the size of the agar pieces plus mycelium should be standardized and removed at the same radial distance from the colony center, to ensure that all have the same amount of mycelium at the same stage of development. When a inoculum from a liquid medium is used, the mycelium can be homogenized aseptically, increasing the number of growing points (Stanbury *et al.*, 1995). However, although quantitative data are unavailable describing the extent of mycelial damage this treatment might cause, anecdotal evidence abounds.

The main aim in using a standardized inoculum is to attempt to achieve process reproducibility, and an active inoculum will decrease the lag phase in subsequent culture. It is essential that the inoculum is transferred at an appropriate time (i.e., when it is in the correct physiological state, which can be determined experimentally).

The effect of inoculum size on performance of *G. lucidum* was investigated by Yang and Liau (1998a). They used an inoculum prepared from a 7-day submerged culture grown in a shake flask. This yielded smaller and more uniformly sized pellets than an inoculum from a slant. Increasing inoculum concentrations increased mycelial yields and numbers of pellets, but pellet was smaller. Fang and Zhong (2002) obtained similar results with *G. lucidum* in shake flasks. When Berovic *et al.* (2003) used 6-day old vegetative inocula from shake flask cultures of *G. lucidum* at concentrations of 14%, 17%, and 20% (wet weight), they noted that the 17% inoculum gave the best growth. With *Co. jiangxiensis*, Xiao *et al.* (2006) reported that mycelial biomass and polysaccharide levels were best with inoculum sizes of 4–6% (v/v), and both fell markedly outside this range. Yet, when Park *et al.* (2001) investigated the effect of inoculum age and volume on growth and EPS production of *Co. militaris*, they found no obvious effects. Lin and Yang (2006) used mycelial suspensions of *Ag. blazei* Murril homogenized with a Waring blender as inoculum in Hinton flasks, and achieved best biomass levels at 11.40 mg/liter inoculum. Their study also highlighted how difficult it is to precisely control inoculum density in different batches and different strains, but, again, why such variability in performance was seen went unexplained.

Thus inoculum concentration may affect mycelial biomass yields and organism morphology, and although the data above are not always easily compared, in general it seems high levels of mycelium production are achieved with small and more uniform-sized pellets, added at high concentrations. Viability tests and especially image analysis seem to possess monitoring features to allow development of more standard and reproducible methods for inoculum preparation, and it is clear that an ability to prepare standardized inocula is a major problem with nonspore forming macrofungi compared to fungi like *Penicillium* and *Aspergillus* spp.

b. Morphology and rheology Both organism morphology and achievable biomass concentrations are influenced by the operating conditions of the fermenter. In turn, the rheological properties of the broths are determined largely by the concentration and morphological state of the mycelium. To control broth viscosity, especially in processes using macrofungi where the viscosity will increase from higher biomass and polysaccharide production, we need to consider the interrelations between the many operating conditions influencing broth viscosity (Olsvik and Kristiansen, 1992).

Macrofungi may grow as a pulpy filamentous form, or as discrete pellets 1–20 mm in diameter (pellet growth). Intermediate forms are also recognized. Some (e.g., Eyal, 1991) describe the submerged culture of macrofungi as usually being characterized by formation of large pellets. These complicate the fermentation process by limiting diffusion of

nutrients into, and wastes out of the pellet, and thus ensures a nonhomogeneous physiological state of the mycelium. In rheological terms, broths behave either as Newtonian or non-Newtonian fluids (i.e., where the ratio between the shear stress and the rate of shear is not constant), depending on the dominant morphological form of the fungus. The filamentous form tends to give rise to a highly viscous, non-Newtonian broth, whereas the pellet form produces essentially a Newtonian system with a much lower viscosity, making oxygen mass transfer rates much faster, at least as far as the pellet surfaces (Kim *et al.*, 2003; Stanbury *et al.*, 1995).

A detailed quantitative structural analysis of fungal morphology is therefore desirable if a better understanding of the relationships between morphology and target metabolite production is sought. This is yet another example of where application of techniques like image analysis would allow the measurement of pertinent morphological parameters (Riley *et al.*, 2000), in combination with rheological analysis. However, as mentioned already, the relationship between fungal morphology and metabolite productivity is still controversial. ''Pellet'' form appears to be preferred in most cases (Gehrig *et al.*, 1998; Hwang *et al.*, 2004; Kim *et al.*, 2003; Lee *et al.*, 1999; Sinha *et al.*, 2001), and especially for EPS formation. Pellet morphology is normally described in terms of shape, circularity or diameter, or surface area, its hairiness or roughness (R), according to the following equation:

$$R = (\text{pellet/aggregate perimeter})^2/(4\pi \times \text{pellet area});$$

and compactness of a pellet estimated as the ratio of the project area of the hyphae in a clump to the projected convex area of that clump (Riley *et al.*, 2000). The physiological significance of these characters to the process under consideration is usually scant.

D. Fermentation strategies

1. Batch cultures

Batch fermentation is the simplest and the most traditional technique for growing macrofungi. Their lag phase is especially long, and can last from 2 to 5 days (Cui *et al.*, 2006; Oh *et al.*, 2007), meaning the whole process can last up to 18 days, depending on the strain used. Thus, their specific growth rates may be as much as 10 times lower than in fungi like *Fusarium* spp. or *Penicillium* spp. This is a major factor restricting the commercial exploitation of these organisms. Therefore, culturing macrofungi on an industrial scale is a very challenging process. As argued earlier, all researchers engaged in growing macrofungi in SLF need to maximize the information arising from their batch cultures, to progress in a more systematic and positive fashion. Specifically, progress is required

not only in their quantitative analysis, but also in metabolomic, transcriptomic, and genomic studies, in order to construct a sound and extensive knowledge base. For example, there is a pressing need to understand the causes of the excessively long lag phases typical of macrofungal inocula.

2. Fed-batch cultures

A fed-batch culture is established initially in batch mode, and is then fed according to different strategies, depending on the type of product desired.

Several reports describe submerged fed-batch culture methods with macrofungi (Berovic *et al.*, 2003; Kim *et al.*, 2006; Mao and Zhong, 2006; Tang and Zhong, 2002; Wagner *et al.*, 2004). For example, low initial glucose concentration (10 g/liter) and feeding three different glucose concentrations (10, 25, and 50 g/liter) into culture medium after most of the initial glucose added had been assimilated (day 6) markedly enhanced process efficiency for *Ganoderma resinaceum* DG-6556. Concentrations of mycelial biomass (42.2 g/liter) and EPS (4.6 g/liter) were achieved when 50 g/liter glucose was added (Jonathan and Fasidi, 2001; Kim *et al.*, 2006). This strategy was based on the hypothesis that high glucose concentrations might inhibit the organism's growth if dump fed initially. Jonathan and Fasidi (2001) suggested with no supporting evidence that at these high initial sugar concentrations osmotic pressure of the culture medium might increase and promote catabolite repression, thereby diminishing both EPS and mycelial biomass production. Tang and Zhong (2002) developed a process for simultaneously producing biomass, ganoderic acid, and polysaccharide by pulse feeding lactose solution, when its residual concentration fell to between 10 and 5 g/liter. By optimizing lactose feeding time and rate to be added, highest cordycepin concentrations (420.5 mg/liter) were obtained in a fed-batch process (see details in Section II.C.2.d). These were 70% higher than those achievable in batch cultivation of *Co. militaris* (Mao and Zhong, 2006).

The fed-batch mode represents a useful tool to study substrate limitation effects, and possible inhibition phenomena (Papagianni, 2004). However, kinetic analysis under these conditions is needed to establish any possible relationships between growth- and metabolite-specific productivity rates, for example, and such relationships can then be exploited to maintain the fed-batch culture at its optimum specific growth rate (feed rate). Another serious limitation with existing studies in this area is that information on macrofungal morphological profiles under fed-batch culture conditions is very limited. Furthermore, in most publications, the feeding strategies used are either not discussed in sufficient detail, or the underlying logic for the choice of the feeding rate and feeding time is not made clear. In fact, in many instances the choices seem arbitrary and subjective. There is clearly great scope for more sophisticated and closely controlled feeding programs based on feedback parameters, like dissolved

oxygen concentration and pH profiles and effluent gas composition and, of course, using limiting substrate concentrations. For more information regarding fed-batch kinetics and modeling, the reader is referred to McNeil and Harvey (1990) and Stanbury *et al.* (1995).

3. Continuous culture

Continuous culture, especially when used as a chemostat, is ideally suited to growth-related products, such as cell biomass, or IPS. So, this method in principle should be attractive for culturing some macrofungi. Clearly, their low specific growth rates might be an impediment, but continuous culture avoids the prolonged downtime of batch cultures and eliminates lengthy lag phases as all cells are growing exponentially. The usual arguments deployed against continuous culture, including increased contamination risk and genetic changes in extended cultures are, of course, highly relevant here. However, Rau (2004) clearly showed the potential of such systems. He used an oxygen-limited chemostat with biomass feedback for the production of Shizophyllan by *Sch. commune*, which provides an insight into what can be achieved when using continuous culture for β glucan synthesis. A threefold productivity increase was achieved compared to batch cultivation.

One other attraction with continuous culture systems is that they generate high-quality data which can unequivocally establish cause and effect relationships between culture parameters. Thus, it is a potentially immensely powerful technique for gaining insights into organism physiology and so more use should be made of it with the macrofungi, at least on a research scale.

4. Other strategies

Two other strategies have been used to grow macrofungi. These are static and immobilized cultures. Fang and Zhong (2002) developed a two-stage process by combining conventional agitated culture (first-stage) and static culture (second-stage), enhancing GA production in *G. lucidum*. Consequently, they optimized the liquid static culture stage by developing an unstructured kinetic model, which predicted cell growth, lactose utilization, and ganoderic acid production. They also successfully scaled up the process from a T-flask (working volume 20 ml) to a three-layer static bioreactor (working volume 7.5 ml).

Immobilization of fungal cells is an attractive technique to obtain high cell densities in order to achieve rapid extracellular enzyme production and this technique seems promising for production of ligninolytic enzymes by macrofungi. Rogalski *et al.* (2006) and Yang *et al.* (2000) discuss some of the advantages of such an approach in more detail, including enhanced enzyme productivity and ease of product recovery and reuse.

E. Optimization of culture conditions

Optimization of the culture conditions in reactors can be a difficult, time-consuming, costly, and empirical process if the classical approach of ''change one-factor-at-a-time'' is employed, which also often does not reveal interactions between any of the many operational variables might be. Hence, experimental design is commonly used in biotechnology to optimize processes, providing systematic and efficient means of achieving particular goals (e.g., producing mycelium and polysaccharide) and simultaneously studying several factors (e.g., inoculation density, pH, temperature, agitation and aeration rate, medium composition). Most software packages now available also indicate beforehand the exact number of experiments needed, and consider the effects on outcomes of possible interactions between factors. Since data are analyzed by a formulated model it is possible to confidently predict how a change in process variables within a given range might affect experimental design, and to distinguish true effects from random variation (Esbensen, 2002). Which experimental design to use depends on the objectives of the experiment and the number of factors under investigation, but this is outside the scope of this review.

Many reports on macrofungal fermentation have used experimental design approaches. These are valuable and now almost indispensable tools for dealing with such complex, lengthy and multivariate processes. However, it is essential to keep in mind the importance of the biochemistry and physiology of the organism involved and the many dynamic phenomena that may simultaneously occur when these fungi grow. Equally it is essential for optimization purposes to integrate and articulate this knowledge with the information provided by the mathematical tools made available with experimental design. Given the large amount of data that arise, and the speed with which optimal parameters (factors/variables) can be identified, it is clearly easy to ignore the metabolic mechanisms that might lead to optimal mycelial yields and metabolite productivities, resulting in inadequate scientific understanding and slow progress in reaching reliable and efficient fermentation strategies. Table 2.3 shows a summary of published reports using experimental design methods with macrofungi. One serious commonly reported problem is in the prior selection of suitable ranges for the chosen control factors in the initial experiments. So some researchers use a one-factor-at-a-time method, or arbitrarily adopt the range of tested values for each variable based only on previous experiences, to first investigate and screen for a suitable type and range of variables (Xu *et al.*, 2003). An effective experimental procedure to approach the optimum neighborhood efficiently is to use the ''steepest ascent'' method (Box *et al.*, 1978; Yang *et al.*, 2003). Different softwares can be used in experimental design,

TABLE 2.3 Summary of reported experimental design methods applied in "higher fungi" fermentation

Organism	Response variables	Optimization variables (factors)	Method	Reference
Ag. cylindracea, *Co. jiangxiensis* JXPJ 0109, *Paecilomyces tenuipes* C240	Mycelium yield Polysaccharide production	Concentration of medium components	Taguchi orthogonal array	Kim *et al.* (2005), Xiao *et al.* (2004a),
Cordyceps pruinosa	Mycelium yield Polysaccharide production	Variety of medium components, Inoculum size, medium capacity, dispersant, culture time	Taguchi orthogonal array	Xiao *et al.* (2004b)
Ganoderma sp. WR-1	Dye (amaranth) decolorization	Concentration of medium components	Taguchi orthogonal array	Revankar and Lele (2007)
G. lucidum	Mycelium yield Polysaccharide production	Concentration of medium components	Taguchi orthogonal array, SAM	Chang *et al.* (2006)
Ag. blazei, *Gr. frondosa* GF9801	Mycelium yield Polysaccharide production	Concentration of medium components	RSM (Box-Behnken design), Regression Model	Liu and Wang (2007), Cui *et al.* (2006)
G. lucidum	Mycelium yield Maximum SCOD	pH, temperature	RSM (CCD)	Lee *et al.* (2003)

Auricularia polytricha	Mycelium yield Polysaccharide production	Concentration of medium components	UD	Xu and Yun (2003)
Gr. frondosa	Mycelium yield Polysaccharide production	pH, aeration, and agitation rate	Simplex method	Lee *et al.* (2004)
An. cinnamomea	Mycelium yield	Concentration of medium components, pH	Full fractional design, SAM, CCD	Yang *et al.* (2003)
Agaricus brasiliensis	Polysaccharide production	Concentration of medium components, pH, temperature	Full fractional design	Fan *et al.* (2007)

SAM, steepest ascent method, RSM, response surface method; SCPD, soluble chemical oxygen demand; CCD, central composite design; UD, uniform design.

including MatLab, CAMO, Minitab, and so on. The great strength of all these approaches is that they assume little prior knowledge, but this is also their greatest weakness. Thus, unless the researcher actively investigates culture physiology at the same time, it is possible to achieve a fully optimized process for macrofungi without having learned anything about how they function.

III. PRODUCTS AND APPLICATIONS

A. General comments

The problems with culturing slow growing macrofungi in submerged axenic culture have already been discussed. However, it should be said that some of the strategies employed with some success to overcome these difficulties with the microfungi have not been applied to these organisms, and the technology for their culture is still fairly unimaginative. For example, some of the novel substrate feed systems which show promise with microfungi (e.g., Bhargava *et al.*, 2003, 2005) still await examination. Much of our understanding of the role, for example, of nitrogen in regulating metabolite production in the microfungi (Demain and Vaishnav, 2006), or how enzyme synthesis is regulated by environmental conditions has not been transferred to the cultivation of the macrofungi. Neither have they been subjected to the levels of genetic manipulation that the microfungi have, which has impacted so positively on their industrial exploitation (Adrio and Demain, 2003, 2006).

Furthermore, in almost all such studies either shake flasks (with little opportunity to control or monitor the environment) or CSTRs have been used, and relatively little effort has been directed at seeing whether better results can be achieved with configurations like airlift systems (Rossi *et al.*, 2002; Table II) or novel fermenter designs (Larsen *et al.*, 2004). It is also worth repeating that in almost all cases, the studies so far reported looking at applying the biotechnological potential of the macrofungi are descriptive, and carried out empirically, generating data with little value in explaining the results obtained.

There is no doubt that their full potential has yet to be properly realized, and we still understand comparatively little about their metabolic diversity since so few have been examined carefully, even though most of their fruiting bodies at least, appear to produce compounds of great interest (Oksanen, 2006; Sabotić *et al.*, 2007), including a range of novel anticancer drugs (e.g., Zaidman *et al.*, 2005). We know now that gene expression is often regulated (not surprisingly) quite differently there than in the corresponding vegetative mycelium (Lacourt *et al.*,

2002; Lee *et al.*, 2002). Consequently screening the fruiting bodies may not be an appropriate approach if the aim eventually is to produce that metabolite by controlled submerged culture of the vegetative mycelium.

As mentioned already, there are attractions in growing these macrofungi as axenic mycelial cultures for both biomass and metabolite production. Here we attempt to give a few recent examples of the commercially valuable products potentially obtainable by the submerged cultivation of macrofungi, and their possible applications. In almost all cases these have not yet been exploited on an industrial scale, and so much of this discussion points to the future and not the present. Such products need to compete with those from other sources, either because of their unique properties or because of their higher levels of productivity. Most probably fall into the former category.

1. Biomass production—inoculum for ectomycorrhizae

Legumes have long been inoculated with appropriate strains of *Rhizobium* or *Bradyrhizobium* to ensure that the formation of root nodules (the nitrogen fixing symbiosis), which is so important to crop production, is encouraged as much as possible. Similar attempts have been made to inoculate the roots of valuable angiosperm and gymnosperm tree seedlings with the appropriate fungi for formation of ectomycorrhizal symbiotic associations (early work reviewed by Trappe, 1977; Harvey, 1991), especially if these trees are to be planted in soils devoid of the symbiotic fungi. This situation may apply to those used for reforestation purposes and for exotic timber production (Chen and Seviour, 2007). Such deliberately infected tree roots may also allow the production and harvesting of the corresponding edible fruiting bodies of the ectomycorrhizal fungi, most of which are not readily produced by other means, and so there is some interest in generating ''mushroom orchards'' in this way too, to supply an increasing demand for these as a source of food (Chang, 2006; Yun and Hall, 2004).

The rapid growth of these trees depends on this mycorrhizal symbiotic association, where the fungus allows increased nutrient assimilation (organic and inorganic phosphorus and nitrogen sources) to occur via the extraradical mycelial network formed in the surrounding soil (van der Heijden and Sanders, 2002). Furthermore, the organized fungal sheath or mantle provides protection against root pathogens (Anderson and Cairney, 2007; Buscot *et al.*, 2000; Smith and Read, 1997; Talor and Alexander, 2005). Thus many trees are totally dependent on these mycorrhizal fungi for their survival and rapid growth, especially in soils which would otherwise require fertilizers to supply sufficient nitrogen and phosphorus.

a. Host specificity A large number of different fungi can form ectomycorrhizal associations with a wide range of plant species, and Talor and Alexander (2005) have suggested that up to 10,000 fungi may possess this ability. Although most are members of the Basidiomycota, the diversity of ectomycorrhizal Ascomycota, about which we know relatively little, is probably much greater than once thought, and some appear to form both ericoid and ectomycorrhizas (Egger, 2006). Clearly any deliberate inoculation program must be based on a sound understanding of host plant–fungus specificity, to ensure that the appropriate fungus–host plant combination is chosen.

Attempts in the past to determine this have been based on identifying the basidiocarps and ascocarps appearing under individual trees, or trying to follow hyphal connections from these to the ectomycorrhizas in the soil (Lakhanpal, 2000). However, sporocarps are not always produced, and tracing such connections is only practically feasible with abundant or visually distinctive (e.g., colored) hyphae. PCR-based molecular methods have now revolutionized our abilities to identify the fungal partner in mycorrhizal communities (Anderson and Cairney, 2007; Dickie and Fitzjohn, 2007; Martin 2007). Several markers have been used (Anderson and Cairney, 2004), including sequence analyses of 18S rRNA genes and internal transcribed spacer regions (ITS) from DNA extracted from mycorrhizal mantle root tips (Nilsson *et al.*, 2006). Saari *et al.* (2005) have described an elegant method involving PCR amplification and sequencing of the ITS to identify the fungus, and microsatellite loci to identify the plant, in a single DNA extract. Fluorescence *in situ* hybridization (FISH) is an attractive means of determining host/fungus specificity and has been use successfully with soil communities, but evidence suggests that probe permeability might be a problem for this.

The molecular data so far obtained suggest that ectomycorrhizal communities may be complex and that several different species or genetically different members of a single species (genets) may infect the roots of a single tree (Anderson and Cairney, 2007). Equally, a single ectomycorrhizal genet can apparently be associated with multiple hosts (Lian *et al.*, 2006) It appears that the host range varies considerably for different fungi (Peter, 2006; Talor and Alexander, 2005), and some like *Pisolithus* can infect roots of many different plant species (explained later).

b. Methods for inoculum preparation Choosing which fungus to use for seedling inoculation will depend on several factors other than its host range. In particular, its ability to grow and form ectomycorrhizas under the same environmental stresses present in the soil (e.g., low soil pH encountered in degraded soils often chosen for reforestation) and under drought conditions (Valdes *et al.*, 2006) may determine the eventual success of any such program. Tolerance to changes in soil CO_2 levels

may also be important (Fransson *et al.*, 2007). The process of root colonization by ectomycorrhizal fungi is clearly a complex and still poorly understood process, involving ''helper bacteria'' and other fungi (Aspray *et al.*, 2006; Garbaye, 1994; Leake *et al.*, 2003) and so inoculation with an appropriate fungus may not necessarily guarantee success (Harvey, 1991; Trappe, 1977).

Several methods for inoculation have been explored (Harvey, 1991; Lakhanpal, 2000; Trappe, 1997). Each has its attractions and limitations. They differ in their level of technological sophistication and required expertise of the staff involved, and include:

a. using soil removed from location of the infected tree roots which is added to ground where seedlings are planted, but may involve moving large amounts of soil long distances, and the risk of transfer of root borne pathogens;
b. using ascospores or basidiospores, or homogenized sporocarps, but because it depends on the level of spore (and sporocarp) production, and their survival after an inevitable storage period (both are often available seasonally) is limited to small-scale inoculation programs. Spore inocula also carry the risk that they fail to germinate once added to soil/roots, but are attractive for less advanced countries (Chen *et al.*, 2006; Lu *et al.*, 1998);
c. using pure cultures of the ectomycorrhizal fungi grown under controlled conditions in submerged culture, which generates inoculum free from contamination produced under controlled conditions. However, this approach is not without criticism either. For example, many of the ectomycorrhizal fungi cannot be grown yet in axenic culture, and as mentioned already, most of those that do grow very slowly and in the form of pellets, making inoculum application problematic on a large scale. Furthermore, for production levels necessary for large-scale inoculation programs, these fungi need to be grown in industry-scale reactors, not in shake flask culture, which most published studies have used. Consequently, much still needs to be learned about how they might behave under such conditions, and how suitable such costly and complex systems are for programs in less well developed countries where reforestation is especially urgent, is an unanswered question.

However, the influence of culture parameters like medium composition, pH, or temperature on the behavior of several ectomycorrhizal Basidiomycota in submerged batch culture has been described with one intention only, that of optimizing their biomass production, and not necessarily of understanding their physiology/biochemistry (Guidot *et al.*, 2005; Job, 1996; Kuek, 1996; Sawyer *et al.*, 2003; Yamanaka, 1999). No published data have used continuous culture techniques for this, where biomass

productivity is much higher than in batch culture, probably because most grow as pellets. Alternatively, these culture studies may provide clues on whether these fungi may tolerate the soil/root conditions (phosphorus levels, pH, pCO_2) encountered following plant inoculation, and successfully form symbiotic associations. It could be argued that such culture conditions bear little relationship to those likely to be found in soil, and so these and other similar studies elucidating their physiology may not help in understanding the process of mycorrhizal establishment and fungal growth *in vivo*. However, some argue persuasively that useful ecological information can emerge from studies of this type (e.g., Fransson *et al.*, 2007). Not too surprisingly, the studies which have been published reveal few general common trends, but instead they seem to emphasize how much still needs to be done in this field, since relatively few media and culture conditions have so far been employed.

c. Application of inoculum To increase the chances of successful inoculation, it is important that the fungus and plant roots are allowed to come into intimate contact. Several methods have been tried with mycelium produced in submerged culture. These include harvesting and homogenizing the mycelium, packaging it in sterile polypropylene bags and burying these in the soil or using a particulate inoculum by allowing the mycelium to first colonize sterile cereal grains or Vermiculite (Lakhanpal, 2000). These methods share common problems of scale up and producing a standardized inoculum, and the risk of carry over of medium nutrients likely to affect root colonization. So other methods for preparing inocula have been described. Thus, some success has been claimed for hydrogel bead inocula, where such concerns no longer apply, and where the cultured mycelium is immobilized in a calcium alginate gel matrix (Kuek *et al.*, 1992). However, these and other methods have not been adopted on an industrial scale, and although several ectomycorrhizal fungi have been grown in submerged culture, the literature suggests that only inoculum of the basidiomycete *Pisolithus tinctorius*, a fungus with a wide host range, is prepared by submerged fermentation (Harvey, 1991). Spore inocula appear to be most popular.

2. Production of polysaccharides—the β-glucans

Members of the Mycota including the fruiting bodies of the Basidiomycota and Ascomycota contain β-glucans (homopolymers of glucose) and other polysaccharides as major structural cell wall components. Their corresponding mycelium may also produce these often in large amounts exocellularly, under appropriate culture conditions. However, this is not always the case (Kues and Liu, 2000), possibly because the conditions necessary for their production have not been elucidated and applied. The β-glucans will be discussed here for reasons which become clearer

later. Their relationship to the production of the corresponding cell wall β-glucans in the producing fungus is also not clear, especially, since the precise chemical nature of many described in the literature is still poorly understood, and commonly a superficial characterization often means that little more than their monomer composition is known.

These β-glucans generally consist of a backbone of glucose residues joined by β-(1,3) glycosidic linkages to which are attached β-(1,6) linked glucose units (Seviour *et al.*, 1992). However, the extent of this branching can vary considerably. In several macrofungi (e.g., *Botryosphaeria rhodina*, *Pleurotus eryngii*, and *Pleurotus ostreatoroseus*, *Sch. commune*; *L. edodes*), the branching frequency is one single glucose residue attached to one in three backbone residues, while in others like *Pestalotia* spp. the branching frequency is much higher with three single β-(1,6) linked glucose units attached every five backbone residues (Misaki *et al.*, 1993). With some, for example, *Botryosphaeran*, their structures appear to vary (Barbosa *et al.*, 2003; Corridi da Silva *et al.*, 2005), depending on culture conditions. This branching frequency shows further variation among the microfungi (e.g., Schmid *et al.*, 2001), and other chemically different glucans are produced by the macrofungi. Thus, β-(1,3)-(1,4)-glucans are detectable in some basidiocarps (e.g., *Collybia dryophila* and other Basidiomycota; Pacheco-Sanchez et al., 2006), as are a range of other polysaccharides including diverse proteoglucans (Wasser, 2002).

It is now clear that many of these fungal β-glucans impact positively on our health. More than 600 species of the Basidiomycota produce basidiocarps which are claimed to have anticancer properties (Reshetnikov *et al.*, 2001; Wassser, 2002). Asian societies have traditionally harvested these both for eating and preparing traditional medicines, but only quite recently have the antitumor properties of these been shown to be due mainly to β-glucans and their derivatives (Cohen *et al.*, 2002; Wasser, 2002; Zaidman *et al.*, 2005). These are also effective in treating a range of infectious microbial diseases in humans and among other attributes, in reducing cholesterol levels and blood pressure (Chen and Seviour, 2007). Lentinan from basidiocarps of *L. edodes*, schizophyllan (SPG, sizofilan or sizofiran) from *Sch. commune* and krestin (PSK) a proteoglycan from *Coriolus* (*Trametes*) *versicolor* have all been approved in Japan for clinical treatment of several cancers and Wasser (2002) claims that about 25% of the funds spent on cancer treatment there is for PSK. The proteoglucan PSP also from *Co. versicolor* is more widely used in China to treat cancers (Wasser, 2002). However, most of the other macrofungi forming biologically active antitumor compounds have not yet been exploited to a similar extent.

a. Mechanism of action of fungal β-glucans as immunomodulators These β-glucans and their chemical derivatives are powerful immunomodulators affecting both our innate and adaptive immune systems. In other

words, they do not attack and kill cancer or microbial cells directly, but act by stimulating our immune system which recognizes β-glucans as foreign or nonself molecules (Chen and Seviour, 2007). It seems that the whole immune system is stimulated, and extensive evidence is now available to indicate that that several cell surface receptors are involved. These include dectin-1 (a lectin), which possesses a carbohydrate (ligand) recognition domain (CRD) binding specifically to β-(1,3) glucans, activating several signaling pathways which promote a variety of immune responses in a manner not yet fully understood (Brown, 2006). Other β-glucans receptors are thought to include complement receptor 3 (C3), scavenger receptors, lactosylceramide and the toll-like receptor (TLR) (Chen and Seviour, 2007). It seems that a single soluble β-(1,3) glucan may activate several different receptors, and different immune cell populations through specific receptors, although several of these may coexist on a single cell type. Much is still to be understood about this ligand–receptor process.

b. β-glucans from fruiting bodies of macrofungi As mentioned above, consumption of fruiting bodies or their extracts has now become an accepted method for helping treat several human diseases in some countries. Wasser (2002) describes methods for extraction and fractionation of the polysaccharides they contain using different solvents, and discusses how diverse the chemical composition of each of the fractions can be among the macrofungi. After screening their bioactivities, it is then possible to hypothesize what structural features enhance or otherwise their antitumor activities. Subsequent chemical modification of these often further enhances this. All the evidence suggests that individual β-glucans can differ markedly in their effectiveness as immunomodulators, even if their superficial structures appear similar. This may be because other physical features are also important, or that we are unable to resolve subtle differences in their chemical composition with the analytical methods currently in use. Nevertheless, there seems to be general agreement that the following polysaccharide properties are needed for high bioactivity (Chen and Seviour, 2007; Kulicke *et al.*, 1997; Wasser, 2002; Zekovic *et al.*, 2005). However, some controversy still exists in the literature, possibly because of inadequate initial chemical characterization or contamination of the glucan examined. This may be the case particularly with structurally complex heteropolysaccharides extracted from fruiting bodies:

- a β-(1,3) linked backbone is essential as α-glucans are biologically inactive,
- β-(1,6) linked side chains increases,
- higher branching frequency increases,

- water solubility increases,
- high molecular weight increases,
- branch modification (e.g., by carboxymethylation) can increase,
- triple helical conformation increases,

Claims that polysaccharides other than the β-glucans or their derivatives from fruiting bodies also have antitumor and other properties (Wasser, 2002), have not always been examined thoroughly.

c. β-glucans produced from mycelial cultures of macrofungi The advantages of growing these macrofungi under controlled conditions as mycelia in submerged culture instead of using their fruiting bodies as the source of the β-glucans have been stated before, but are worth repeating. Large-scale production facilities and axenic cultures grown under carefully monitored and controlled conditions guarantee a more reliable product of known composition. They thus provide an opportunity to optimize yields and possibly manipulate the chemical and physical properties of the polysaccharides to improve their bioactivities. Furthermore, as most are synthesized exocellularly, their recovery is much simpler too. However, the eventual exploitation of such processes will be determined by their productivity and biological effectiveness, and competitiveness with those from other fungal sources. Also several examples exist where the chemical and biological properties of the polysaccharide produced in submerged culture are quite different to those synthesized by the fruiting body, and in some cases no polysaccharide is produced or they lack any biological activity because of structural differences (Wasser, 2002).

Some examples where macrofungi have been grown in submerged culture for exocellular polysaccharide (EPS) production are given in Table 2.4. These illustrate many of the limitations mentioned earlier in this article in studies with the macrofungi. They reveal that only a comparatively few fungi have been assessed in this way, that many studies use shake flask culture where monitoring and control of culture parameters are not available, and are usually descriptive. From these published data, few general principles as to how EPS yields might be increased to a level attractive for commercial exploitation emerge. Consequently, basidiocarps will probably still remain the main source of lentinan in the immediate future, and all the attractions of using ''pure'' EPS sources not realized.

3. Macrofungi as sources of enzymes

In general, fungi have a quite remarkable ability to synthesize and secrete enzymes capable of degrading complex polymers to low-molecular-weight products which they then can assimilate and feed on. Consequently, they play vital roles in the carbon cycle, for example, in

TABLE 2.4 Recent examples of studies using macrofungi for EPS production

	Fermentation system	Chemistry of EPS	Comments	Reference
Ascomycota				
B. rhodina	Shake flasks	β-(1,3)-(1,6) Glucan	Determined effect of pH and nitrogen source on biomass and EPS yields, with optimal conditions at pH 3.7 and 2% $NaNO_3$	Selbmann *et al.* (2003)
B. rhodina	Shake flasks and lab-scale reactors of different configuration	β-(1,3)-(1,6) Glucan	Higher yields with CSTR fitted with marine propeller and draught tube, and further increase with airlift reactor with external loop. Shake flasks gave best yields of all	Selbmann *et al.* (2004)
B. rhodina	Shake flasks	β-Glucan	Determined effect of different carbon sources on yields, and all but mannitol supported EPS production, with sucrose the best	Steluti *et al.* (2004)
B. rhodina	Shake flasks	β-(1,3)-(1,6) Glucan	Branching frequency of EPS affected by carbon source, increasing with fructose compared with sucrose	da Silva (2005)
B. rhodina	Shake flasks and lab-scale CSTR	β-Glucan	Greatest EPS yields achieved under nitrogen and oxygen-limiting conditions, and β-(1,3)-(1,6) glucanase production affected yields	Crognale *et al.* (2007)
Co. militaris	Lab-scale CSTR	Not reported	Low agitation speeds increased EPS but not mycelial production, and image analysis suggested that compact pelleted morphology corresponded to highest EPS production	Park *et al.* (2002)

Co. militaris	Lab-scale CSTR	Not reported	Conditions of pH and temperature favouring optimal EPS yield did not correspond to those favouring biomass production. Image analysis suggested larger and more compact pellets favored EPS production.	Kim *et al.* (2003)
Co. militaris	Shake flasks and lab-scale CSTR	Glucans	EPS production was higher than with Cordyceps sinensis, and achieved under different culture conditions of pH	Kim and Yun, 2005
Co. jiangxiensis	Shake flasks	Not reported	Culture conditions were optimized for EPS production, although EPS yields appear lower than in other Cordyceps. Growth was as pellets	Xiao *et al.* (2004)
Basidiomycota				
Ag. blazei	Lab-scale CSTR	β-Glucan and others	Chemical composition of EPS changed with culture pH, and lower pH favoured β-glucan synthesis. EPS production fell following glucose exhaustion from the medium	Shu *et al.* (2004)
Ag. brasiliensis	Shake flasks	Not reported	Chemical composition of medium (carbon and nitrogen sources) optimized for EPS production and its bioactivity	Fan *et al.* (2003)
		Not reported		

(*continued*)

TABLE 2.4 (*continued*)

	Fermentation system	Chemistry of EPS	Comments	Reference
Auricularia auricula	Shake flasks and lab-scale CSTR		Reported effect of pH on biomass and EPS production, and a 2-stage pH control strategy shown to optimize both	Wu *et al.* (2006)
Ga. applanatum	Shake flasks	Not reported	More EPS production occurred in stationary phase, and its molecular weight increased with incubation time	Young Lee *et al.* (2007)
G. lucidum	Shake flasks and lab-scale CSTR	Not reported	Fed-batch process developed based on shake flask data, and equivalent yields were reported when dissolved oxygen levels were controlled	Tang and Zhong 2002
G. lucidum	Lab-scale CSTR	β-Glucans	Batch and fed-batch systems studied with EPS containing several structurally similar β-glucans	Berovic *et al.* (2003)
G. lucidum	Shake flasks	Not reported	Reported how EPS yields are affected by conditions of nutrient limitation, showing that nitrogen and dissolved oxygen limitations favoured EPS production	Hsieh *et al.* (2006)
Gr. frondosa	Shake flasks and lab-scale CSTR and airlift fermenter	Not reported, but differed in molecular weights	Optimized conditions for EPS and myceial production in the two fermenter configurations. Higher yield in CSTR. Morphology affected by aeration rates, and the best morphology for EPs production were ''feather-like'' mycelial clumps	Lee *et al.* (2004)

L. edodes	Shake flasks and lab-scale CSTR	Not reported	Optimized medium carbon and nitrogen sources for EPS production	Elisashvili *et al.* (2006)
Phellinus gilvus	Shake flasks and lab-scale CSTR	Diverse	Optimized medium composition for EPS production, which were often different to those for mycelial formation. Four EPS fractions were obtained, differing in their chemistry	Hwang *et al.* (2003)
Phellinus spp.	Shake flasks and lab-scale CSTR	Not reported	Optimal pH for EPS production varied with species, and pelleted growth was seen at lower pH. *Phellinus gilvus* producing smooth pellets was highest EPS producer of three species examined	Hwang *et al.* (2004)

transforming plant debris containing cellulose and lignin. The physiologically distinctive group of white rot fungi (mentioned earlier in this review) and containing members of both the *Ascomycota* and mainly *Basidiomycota*, are of special interest. They are so named because they bleach any wood they colonize by degrading the lignin component of the lignocellulosic material there (Cohen *et al.*, 2002; Pointing, 2001). Lignin is a chemically variable but highly recalcitrant complex polyphenolic polymer, but can be readily degraded by this group of fungi, and because of the importance of this process in carbon recycling, its enzymology has been extensively studied. It is now clear that at least one of three (exocellular?) enzymes (the lignin modifying enzymes or LMEs) is involved (Baldrian, 2006; Mester *et al.*, 2004) in lignin degradation:

- a glycosylated heme-containing peroxidase, lignin peroxidase (LiP);
- another glycosylated heme-containing Mn dependent peroxidase (MnP), oxidizing Mn(II) to Mn(III), using H_2O_2 as oxidant;
- a copper containing phenol oxidase or laccase.

As Pointing (2001) points out, lignin oxidation does not provide the fungi with any energy, but serves to make available as energy sources other polysaccharides (cellulose and hemicellulose) in the lignocellulosic material. How these enzymes work is discussed by Pointing (2001), Mester *et al.* (2004), and Baldrian (2006). Their degradative mechanisms involve the generation of free radicals, which are chemically highly reactive, and mineralize lignin. The recently annotated whole genome sequence of the white rot fungus *Pha. chrysosporium* (Kersten and Cullen, 2007) will allow some of the current uncertainty regarding lignin degradation to be clarified.

Submerged culture of some white rot fungi have been used to produce these LMEs and optimize their yields for subsequent testing in bioremediation trials, but again most of this work has been carried out at lab-scale, most often using shake flask cultures. Furthermore, many of the fungal strains used in these studies were obtained from culture collections, and not immediately from fruiting bodies produced in the environment. Examples of recent studies of this type are listed in Table 2.5. Certain features emerge from these. For example, relatively little effort has been devoted to resolve the factors affecting LiP production in these fungi, and most of the work has been directed more at understanding those affecting the yields of MnP and laccase, and in obtaining these enzymes for subsequent purification and characterization. Developing large-scale processes for eventual industrial application has received little attention. Clearly the data show that production of the enzyme can be increased markedly often by manipulation of the medium, and in some of these fungi by adding appropriate inducers like ethanol, and manipulating the growth conditions. The reasons why these events occur are not always

TABLE 2.5 Examples of recent studies into production of LMEs produced by macrofungi

Fungus	Culture system	Enzyme/s studied	Comments	Reference
Pycnoporus cinnabarinus	Shake flasks	Laccase	Ethanol induces laccase production	Lomascolo *et al.* (2003)
Ganoderma spp.	Shake flasks	LiP MnP and laccase	Presence of wheat bran increased enzyme yield but differences in enzymatic activity between the two strains	Silva *et al.* (2005)
Pleurotus dryinus	Shake flasks	Laccase and MnP	Grown on mandarin peel and tree leaves; addition of Cu and Mn increased yield of both enzymes, and Mn addition affected relative amounts produced; lignocellulose required for MnP production	Elisashvili *et al.* (2006)
Pl. ostreatus	Shake flasks	laccase	Genetically modified strain with a recombinant mnp2 sequence overproduced the laccase	Tsukihra *et al.* (2006)
Pha. chrysosporium	Shake flasks and laboratory scale stirred-tank reactor	LiP	Higher yields obtained in controlled conditions compared to shake flask culture, and yields related to pellet formation	Michel *et al.* (1990)
Various strains of Basidiomycota	Shake flasks	MnP and laccase	Expression of enzyme activities varied between species and strains. Different lignocellulosic substrates gave different enzyme yields, and nitrogen source affected rates of production	Songulashvilli *et al.* (2006)
Pleurotus spp.	Shake flasks	Laccase,	Best laccase yields with mandarin peel and grapevine dust, with ammonium sulphate	Stajić *et al.* (2006)

(*continued*)

TABLE 2.5 (*continued*)

Fungus	Culture system	Enzyme/s studied	Comments	Reference
			as nitrogen source. Yield especially of peroxidase lower than with solid-state fermentation systems	
Funalia trogii and *Tra. versicolor*	Shake flasks	Laccase	Recycling pellets of both fungi in Cu supplemented medium increased laccase production in both these fungi	Birhanli and Yesilada (2006)
Various strains of Basidiomycota	Shake flasks	Laccase	Ferulic acid and mannan oligosaccharide elicitor added singly and together markedly enhanced laccase in *Pycnoporous sanguineus*, *Coriolopsis polyzona*, and *Pl. ostreatus*	Vanhulle *et al.* (2007a,b)
Cl. dusenii and *Hypholoma frowardii*	Pilot plant bioreactors (up to 300l)	MnP	High yields were obtained with acetate as carbon source and Mn supplementation. Scale up did not affect MnP yields	Nüske *et al.* (2002)
Tra. trogii	Shake flasks	Laccase, MnP	Yields increased by addition of Cu and with low nitrogen (asparagine) content	Trupkin *et al.* (2003)
Fomes sclerodermeus	Shake flasks	Laccase and MnP	Laccase and MnP induced by Mn in the medium, while Cu had a negative effect on MnP production	Papinutti and Forchiassin 2003
Several white rot fungi	Shake flasks	MnP	MnP induced by several lignin preparations and veratryl alcohol and guiacol.	Gill and Arora 2003
Tra. trogii	Shake flasks	Laccase	Sole lignolytic activity was due to laccases whose production was increased by addition of Cu	Zouari-Mechichi *et al.* (2006)

examined and the different fungi respond in different ways to these influences. Comparatively few studies have worried about better understanding the molecular bases for regulation of LME synthesis. It is possible that any future industrial applications will use purified (and immobilized) enzyme preparations obtained from large-scale biomass production (Robinson *et al.*, 2001), but much still needs to be learned about the processes involved in their production.

4. Applying macrofungi in bioremediation processes

All three LMEs are highly nonspecific in terms of which substrates the radicals they generate can attack. Hence, the white rot fungi producing these can also metabolize a range of other substrates, including xenobiotics, some which pose serious threats to the environment (e.g., the LME producing fungi are the only organisms capable of metabolizing some polyaromatic hydrocarbons or PAHs). Potentially their exploitation in bioremediation processes is therefore considerable (Couto and Toca Herrera, 2006; Pointing, 2001). Thus, white rot fungi and/or their lignolytic enzymes are known to detoxify a range of industrial effluents, and degrade a range of pesticides, polychlorinated biphenyls, and synthetic plastics, and especially synthetic dyes used in the textile and other industries. This latter application has attracted the most attention, since these dyes are produced in enormous amounts globally, and some are both mutagenic and carcinogenic. Furthermore, they are not efficiently removed by conventional wastewater treatment processes, and thus pose a serious risk. Nonbiological treatment methods are expensive and generally perform poorly too. Although most of the early work on dye reduction used either *Pha. chrysosporium* or *Co. versicolor*, it is quite clear now that many white rot fungi possess a similar ability, and some perform better. As with most other studies with macrofungi, the extensive published data are still largely preliminary, and most have been generated from laboratory scale experiments. Examples of a few more recent publications are given in Table 2.6, and much more extensive lists are provided by Couto and Toca Herrera (2006).

One important point to emerge from these and the many other similar studies is that the enzymes responsible for dye decolorization are not necessarily restricted to the LMEs, and that others may play important if yet poorly understood roles. In addition, not all fungi respond identically to similar culture conditions and so, for example, although many produce more lignolytic enzymes and decolorize dyes faster under nitrogen limiting conditions, some perform better in nitrogen-rich medium. However, bearing in mind the current levels of publication in this field, there seems to be considerable interest in eventually using these white rot fungi, or the harvested enzymes they synthesize, to treat such hazardous wastes

TABLE 2.6 A few recent studies using white rot fungi to decolorize synthetic textile dyes

Fungus	Culture system	Comments	Reference
Bjerkandera adusta, *Phlebia tremellosa*	Shake flasks Whole fungi	Nitrogen limiting conditions did not improve decolorization rates	Robinson *et al.* (2001)
Ischnoderma resinosum	Shake flasks with whole organism and purified laccase and MnP	Purified MnP decolorized all the dyes examined while purified laccase was more selective. Culture medium with crude enzymes also worked with all the dyes, especially at low pH	Kokol *et al.* (2007)
Several Basidiomycota	Shake flasks Whole fungi	Agitated cultures growing as beads were more efficient than static cultures, and *Ganoderma australe* grown in nitrogen rich medium performed best	Rigas and Dritsa 2006
Pycnoporus sanguineus	Shake flasks Whole fungi	Laccase production increased in presence of these dyes but evidence suggesting other enzyme (cellobiose dehydrogenase) activity involved in a positive and negative manner in this process	Vanhulle *et al.* (2007a,b)
Fu. trogii	Shake flasks Whole cells and immobilized cells	Three LME' were involved in this process and decolorization occurred more rapidly with immobilized cells than with those in suspension	Park *et al.* (2006)

Several white rot fungi	Shake flasks	*Tra. versicolor* was the best strain examined and the anthraquinonic dyes were most 3easily decolorized. No LiP activity detected during process, and laccase alone was not successful in decolorization	Liu *et al.* (2004)
Tra. versicolor	Rotating biological contact (RBC) reactor operating in batch and continuous mode	More rapid decolorization in the RBC than in a stirred-tank reactor, and oxygen saturation conditions gave best results with N-free medium	Ramsay *et al.* (2006)
Several white rot fungi	Shake flasks	Under nitrogen limiting conditions, some performed better than *Pha. chrysosporium* with one or all of the dyes	Chander *et al.* (2004)
I. resinosum	Shake flasks	Higher capacity than other fungi examined and not affected by different levels of nitrogen in the medium	Eichlerová *et al.* (2006)
Tra. versicolor	Air pulsed fluidized bioreactor operating in batch and continuous mode	Pelleted growth and under continuous conditions, decolorization affected by cell retention time in the reactor	Blanquez *et al.* (2006)

(Couto and Toca Herrera, 2006; Robinson *et al.*, 2001), because they offer potential advantages over other current technologies.

IV. CONCLUSIONS

Traditionally, mankind has harvested the sporing bodies of the macrofungi as sources of food, nutrients, and medicines. However, the submerged liquid culture of the vegetative or mycelial forms of these fungi offers considerable biotechnological potential in terms of delivering biomass, and bioactive metabolites for therapeutic purposes, or in applications such as bioremediation and detoxification, and environmental improvement. Several challenges still stand in the way of our fully developing the potential of these promising cultures, some of which have their roots in their complex growth characteristics, but many of which relate simply to the undeveloped nature of the science and engineering knowledge base relating to these fungi. There is a pressing need to achieve better understanding of the fermenter cultures of macrofungi. The best approach to this would be to use a combination of advanced experimental design, analytical science, appropriate reactor technology, and modeling to increase our process physiology knowledge base.

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CHAPTER 3

Bioprocessing Using Novel Cell Culture Systems

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1. INTRODUCTION

Plant cells and plant tissue cultures have long been considered as attractive systems for expressing secondary metabolites and recombinant proteins, including glycosylated proteins, for commercial production and biopharmaceutical applications. More recently, with advances in molecular biology and genetics, increasing interest is being focused on plant-based systems, particularly on plant suspension cell fermentation in bioreactors, for the production of high-value recombinant proteins (Doran, 2000). While heterologous proteins can be produced in bulk in alternative expression systems such as bacteria, yeast, and mammalian cell cultures, use of transgenic plant cell cultures offers significant advantages. Compared to microbial cells, the posttranslational processes, such as propeptide processing, signal peptide cleavage, and glycosylation for expressing functional protein in plant cells are similar to the posttranslational processes for mammalian cells (James and Lee, 2001). Bacteria cannot perform the posttranslational modifications typical of eukaryotes and thus do not allow the production of glycosylated full-size functional proteins. In addition, bacterial cells contain endotoxins that are difficult to eliminate during purification. Plant cells have the capability to fold and assemble complex multimeric glycosylated proteins that are more similar to mammalian cells (Ma *et al*., 2003). Furthermore, there are other intrinsic features of plant suspension cells that make them particularly attractive for the production of foreign proteins; namely avoiding the use of animal-derived material (Su, 1995), using simple culture media primarily consisting of inorganic salts, sugar, and simple organic-acid growth regulators. Plant cell cultures are also easily scalable to very large-scale production. Fermentors of up to 75,000 liter capacity have been used for paclitaxel production (Phyton Biotech: www.phytonbiotech.com). Finally, recombinant proteins produced by bacteria often form inclusion bodies which create the possibility of incorrect protein folding, making labor and cost intensive *in vitro* refolding necessary (Fischer *et al*., 2004).

Although mammalian cells are well-established systems for recombinant protein production, some drawbacks to mammalian cell hosts are well known (e.g., high cost due to expensive medium and complicated purification process, slow growth and potential for contamination by mammalian pathogens from animal sources) (Parekh, 2004). In downstream processing, care must be taken to remove oncogenic sequences or viral contamination, if *in vivo* use is intended. Additionally, the use of transgenic animals as a source of recombinant proteins is becoming more and more limited because of legal and ethical restrictions. In contrast, plant cell culture systems have low raw material cost requirements and are naturally incompatible hosts for animal viruses. Like mammalian cells, proteins can be readily engineered to be secreted into the liquid medium, further reducing the purification costs (Su, 1995).

The large-scale production of recombinant antibodies and antibody fragments using whole plants in the field is a well-established technique in recent years (Fischer *et al*., 2004). However, climatic variations create uncertainty of sustained supply from open field cultivation. Other concerns of transgenic crops, including environmental risk of transgenes spread, contamination of food source, and evolution of new and uncharacterized strains or species, create a higher regulatory barrier to product approval (Gupta and Ram, 2004).

Unlike transgenic plant-based open field production systems, plant cells when propagated in a confined environment (i.e., fermentation in a flask or a bioreactor system) offer greater advantage because recombinant therapeutics can be produced under controlled conditions eliminating the risk of escape of bioengineered genes with pollen or seeds (Parekh, 2004). Moreover, it takes less than 6 months from the time of gene introduction to protein production at usable levels compared with the 12–16 months (Table 3.1) that it takes to produce the same amount of recombinant proteins derived from GMO-based whole plant systems (Sharp and Doran, 2001). Additionally, the parameters in the fermentor related to biomass accumulation and transgene expression can be optimized. Therefore, being independent of geographical location, climate, season, and other factors that affect traditional cultivation methods of whole plants, *in vitro* production using plant cell fermentation under controlled conditions improves the speed and reliability of the product supply to the market.

For over three decades, secondary metabolite productions in bioreactors have used the process of large-scale plant cell fermentation (Scragg, 1992). These metabolites include alkaloids, antibiotics, volatile oils, resins, tannins, cardiac glycosides, sterols, and saponins. Success in large-scale culture of secondary metabolite biosynthesis is limited because the cultures exhibit relatively slow rates of growth, and the biosynthesis of the desired compound is often at a much lower level than in the intact plant (Misawa, 1985).

TABLE 3.1 Typical production time for producing therapeutics derived from various transgenic systems

GMO	Transgenic production
Transgenic animals (farm animals)	2–3 years
Transgenic mammalian cell culture	6 months
Transgenic crops (tobacco and corn)	0.5–1 year
Insect cells culture	1–2 months
Fungi (*Aspergillus*—yeast)	2 weeks
Bacteria (*Escherichia coli*)	1 week

A number of plant species have been used for recombinant protein production using cell fermentation, ranging from model systems like *Arabidopsis*, *Catharanthus*, and *Taxus*, to important monocotyledonous or dicotyledonous crop plants like rice, soya bean, alfalfa, and tobacco (Petolino *et al.*, 2003; Scragg, 1992). These plant cell culture systems have successfully demonstrated the capability to produce a variety of recombinant proteins (i.e., antibodies, vaccines, and other therapeutic molecules) in either shake flask or in small bioreactors. Of the various plant cell systems used for *in vitro* cultivation, such as hairy roots, immobilized, and free cell suspensions, the latter is generally regarded to be the most suitable for large-scale applications in the biotechnology industry (Scragg, 1998). However, compared with the more conventional expression systems like bacteria, yeast, and mammalian cell culture, the number of applications are still relatively low (Tables 3.2 and 3.3). Although plant cells offer a unique combination of physical and biological properties, due to the inherent tightly controlled gene and protein expression machinery, it is more difficult to manipulate and tweak the expression system in plant cells. Reported yields of protein range 0.1–50 mg/liter of culture, although a product level as high as 200 mg/liter has been reported in transgenic rice suspension culture (Fischer *et al.*, 2004; Huang *et al.*, 2001). Despite these developments, the use of plant cell culture for the large-scale production of recombinant proteins is still in its infancy. It is anticipated, however, that advances in the genetic engineering of plants and the demand for large quantities of new or improved diagnostics and therapeutics in the healthcare and pharmaceutical markets will lead to a rapid expansion in this research field.

Plant cell culture processes employed for secondary metabolite or recombinant protein production resemble conventional fermentation processes. In principle, the fermentation of plant cells requires similar techniques and equipment as that of microbial fermentation. However, it should be emphasized that there are distinctive properties associated with plant cells that call for unique strategies in designing and implementing plant cell fermentation processes (Table 3.4).

The upstream process is normally initiated from plant calli (dedifferentiated or differentiated tissue) cultivated on solidified media, followed by a transfer to liquid medium for growth in shake flasks or in bioreactors. Progression into the fermentation process over time results in the formation of small aggregates, of $<$10–50 cells sometimes measuring a millimeter or more, in the culture. Plant cell cultures are not comparable in terms of generation times to other cell culture systems in terms of obtainable cell densities or nutritional requirements. Nevertheless, it is possible to cultivate plant cell suspensions using conventional fermentation equipment with minor adjustments and to apply standard modes of fermentation operations like batch, fed-batch, perfusion, and continuous fermentation

TABLE 3.2 Advantages and disadvantages with various GMO cell cultures

GMO	Advantages	Disadvantages
Bacteria	Inexpensive media	Certain proteins not secreted
	High expression level	Protein aggregation
	Fast growing	Contains endotoxins/ pyrogens
	Easy to characterize	No posttranslation modification
	Established regulatory track record	Possibility of incorrect folding
Mammalian	Can secrete proteins	Expensive media
	Correct folding	May contain allergens and animal-derived components
	Posttranslation modification	Requires extensive characterization
	Good GMP track record	May contain adventitious material
Insect cells	Product is secreted	Minimum regulatory record
	Baculovirus are harmless to humans	Incorrect protein glycosylation
	Proper posttranslation modification	Expensive media
	High expression level	Mammalian virus can infect cells
		Short history of use
Yeast	Recognized as ''safe''	Certain over-glycosylation can alter bioactivity
	Long history of use	May contain immunogens
	High expression level	
	No endotoxin	
	Rapid growth	
	Inexpensive media	
	Protein mostly folded correctly	

(Sajic *et al.*, 2000; Scragg, 1992). Large-scale fermentations (Table 3.5), up to a volume of 75,000 liters, have been performed successfully with plant cell systems (Taticek *et al.*, 1994).

The feasibility of the fermentation approach has been confirmed by large-scale production of several high-value recombinant proteins in plant cell cultures (e.g., tobacco cell culture). Recent improvements in

TABLE 3.3 Comparison of the advantages and disadvantages of animal vs. whole plant expression systems

	Animals	Plants
Advantages	High expression levels	Shorter cycle than animals
	Can process complex proteins	Easy storage of seed bank
	Correctly fold proteins	Low-cost production
	Easy scale-up	Well-understood genetics
	Low-cost production	
Disadvantages	Little regulatory experience	May contain allergens
	Variable expression level	Different posttranslation modification made by animals
	Complicated definition of lots and batches	Unresolved public acceptance issues
	Long time-scale	Potential source of herbicide, pesticide impurity
	Unresolved public image	

the design of novel promoters (Shrawat and Lorz, 2006) and other control elements, in combination with deeper insights into the mechanism of plant gene silencing (Hiei *et al*., 1994) and gene targeting, will lead to significant improvements in product yield. This will lay the basis for predicting the behavior of transgenes in plant cell cultures. The advent of new transformation technologies will allow us to extend production to a broader range of plant species that have better characteristics for protein expression than does tobacco (Table 3.4). A systematic evaluation of growth and induction conditions should result in higher productivity and more applications of plant cell suspension culture in recombinant protein production.

At present, there are many issues that must be addressed in order to successfully scale-up the cultivation process for recombinant protein production. Briefly, the following issues remain to be investigated: (1) further screening of stable, high-expression cell lines; (2) systematic development of selected cell lines; (3) studies on the physiology and metabolism of cultured cells in bioreactors; (4) optimization of the culture environment in a bioreactor; (5) monitoring, modeling, and control of cultivation processes in a bioreactor; (6) investigation of bioreactor

TABLE 3.4 Characteristics desired in transgenic plant cell cultures

Cell biology	Product expression	Recovery and isolation	Safety/quality
Rapid growth	At high levels	In supernatant (extracellular)	No adventitious agents (fungi, bacteria)
Animal protein free medium	Control of product expression	Stable	No oncogenic DNA
Few growth factors	Stable productivity	No interfering proteins	
Protein processing and transport close to human cells	Efficient and accurate post translation modifications	Few steps for recovery	
		Less sensitive to degradation	

TABLE 3.5 Challenges with the scale-up of plant cells

Sensitivity to hydrodynamic shear stress (agitation and aeration): case by case
Aggregates, clumps/morphology
Slow growth or loss of viability/stability over time
Sterility
Foam/adhesion to bioreactor walls
Process optimization (micro/macro kinetics)
Viability and productivity at high density
Mass transfer/O_2/mixing homogeneity
Product expression, regulation/translocation
• Inhibitors: co-product formation
Yield Variability
• Product recovery, purity and activity

scale-up; (7) downstream processing; and (8) engineering of plant cells with favorable posttranslational modifications to more faithfully resemble proteins produced in mammalian cells. The majority of research efforts should be addressed towards developing competent cell lines,

and to establish standard protocols for cell line development, cell banking, and cryopreservation.

This review encompasses two parts. The first addresses unique properties of plant cell cultures relevant to upstream process development with specific focus on reviewing the generation of transformed suspension cell lines expressing small molecules and complex recombinant proteins (e.g., antibodies), cell line development, cell banking, cryopreservation, and process development considerations during scale-up. The second part discusses key topics relevant to bioreactor engineering, including process scale-up and bioreactor operating strategies that are critical to successful bioprocess development from plant cell platforms.

II. PLANT CELL CULTURE DEVELOPMENT FOR SCALE-UP

A. Plant suspension culture initiation and establishment

Plant suspension cultures are generally initiated by transferring pieces of undifferentiated callus or embryogenic tissue commonly derived from various explants (e.g., stems, leaves, roots, immature embryos, mature embryos) to a liquid medium, which is then agitated on a shaker during the period of culture (Fig. 3.1). Friable callus is preferred for initiating

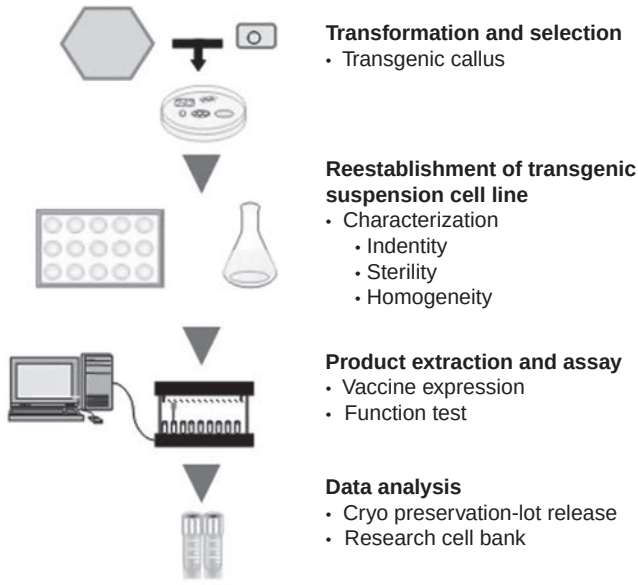

FIGURE 3.1 Suspension cell line development.

suspension cultures as it is easily broken into small pieces under agitation. For some species producing hard type I callus (e.g., rice, turf grasses), several rounds of transfers from liquid media to solid agar media are helpful for obtaining friable calluses. In some instances, adding 2% of coconut water or 5% yeast extract also helped the formation of friable callus (Payne *et al*., 1992). Another method of obtaining suspension cultures of a monocotyledonous species, orchardgrass (*Dactylis glomerata* L.), with small aggregates has been reported by Horn *et al.* (1988). Although a longer time period is required, cell suspension cultures can also be initiated by inoculating the liquid medium with a homogenized explant mixture of differentiated plant materials (e.g., a fragment of a hypocotyl or cotyledon). Thus, the initial suspension has various sizes of differentiated tissue pieces, dead cells, and some undifferentiated cell clumps. The latter will usually divide and gradually free themselves from the mixture, and subsequently could be selected for subculturing (Schemale *et al*., 2006).

For suspension cultures, several doublings are required for the culture to become established, as is evidenced by its consistent growth pattern. An established cell line usually exhibits a rapid rate of growth that is similar from one subculture to the next subculture. Unfortunately, there have been no thorough investigations of characteristics common to the cells and cell clusters in liquid-grown cultures that serve as good sources for transformation and scale-up. Cultures are often described as consisting primarily of small and densely cytoplasmic cells containing a few elongated and highly vacuolated cells. The sizes of cell aggregates range from a few cells per aggregate to more than 100 cells per aggregate. As mentioned previously, the development of suspension cultures appears to represent a continuous selection for a higher percentage of cells that exhibit totipotency. However, an inability to precisely describe these embryogenic suspension cultures and to provide information on their routine development is still a paramount problem in plant cell culture (Staub, 2003).

B. Development of homogeneous cell lines

Homogeneous cell lines are usually obtained by long-term cultivation, along with periodic filtration via meshes and subjecting the cell clusters to optimal agitation speed. Tobacco BY-2 is a well-known homogeneous line. However, it should be kept in mind that no suspension cell line has been shown to be entirely composed of single cells (Petolino *et al*., 2003) because of the failure of cells to easily separate following mitosis. Homogeneous lines are suitable for transformation and cell line development, which results in increased consistency in protein expression. To create a homogeneous cell line, metal meshes with various sizes, starting with the largest mesh size, to filter out large clumps several times may be used.

Washing cells periodically with fresh liquid media also helps to disaggregate the cells. Eventually, the suspension will be composed of single or few-cell aggregates (4–10 cells).

C. Development of single cell-derived cell lines

As soon as a homogeneous cell line is established, the cells can be used to develop the single cell-derived cell line. A single cell-derived cell line has the advantage of genetic stability, rapid growth, and persistent performance; in particular, suitable for developing synchronized cell lines (Schemale *et al.*, 2006). Such a line is acquired by plating the suspension onto agar plates and starting a new suspension from a single aggregate that grows to a colony.

D. Development of synchronized cell lines

The synchronized cell line usually has a high rate of growth and high productivity. Previously, the studies done by Schemale *et al.* (2006) with the synchronized tobacco BY-2 line showed exceptionally high growth rates.

E. Example of plant cell culture—rice suspension cells

Several explants and media have been used for rice suspension cultures. The explants producing the initial callus used for initiating suspension cultures have been leaves, immature embryos, mature embryos, anthers, or inflorescences. A surprising success was achieved with callus derived from mature embryos. It was found that immature embryos produced a more friable callus (Petolino *et al.*, 2003), which ultimately establishes homogeneous cell lines faster.

Many different media are used for *in vitro* culture of rice suspension cultures; the primary references and the composition of nutrient media are compared in Payne *et al.* (1992). Media used for growing the suspension cultures cell include R2, B5, LS, AA, and N6. The nitrogen source has been found to be especially important in the suspension media, and the use of the AA medium, which provides all nitrogen via amino acids, has been found to be effective for suspension culture maintenance. Sucrose has been used exclusively as the energy source and at concentration of 2–3%. The 2,4-D concentrations used in the suspension media are in the range of 0.5–4 mg/liter. Generally, no other hormones are added, although Trexter *et al.* (2002) did include 0.2 mg/liter zeatin along with 1.2–2.5 mg/liter 2,4-D. Also, the AA medium includes 0.1 mg/liter

gibberellic acid (GA_3) and 0.2 mg/liter kinetin. The dilution that occurs at subculture varies from 1 to 4 ml of packed cell volume (pcv) per 10 ml of fresh medium. The suspension cultures are shaken on a shaker between 100 and 160 rpm, with most procedures using 120–130 rpm. Subculturing should occur twice weekly during the initial phase and then once per week after the culture is established. During the subculturing, care must be taken to select for small clusters that are white to cream color, and it is important to avoid clusters that show any necrosis (Payne *et al*., 1992).

Based upon the large number of genotypes that have been initiated, the different explants used, and the different media employed, the key to developing effective suspension cultures appears to be more related to the cell selection process at the time of subculture than to any other factors. Because this selection process tends to be a highly visual and subjective, it is difficult to describe in such a way as to make it easily reproducible. Additional research on the characterization and identification of unique morphological or biochemical features of suspension cultures is needed to make their development more precise.

After a short time, the culture will be composed of cellular aggregates of varying size, residual pieces of the inoculum, and the remains of dead cells. The term ''friability'' is used to describe the separation of cells following cell division. Formation of a ''good suspension,'' a culture consisting of a high percentage of small aggregates, is much more complex than finding the optimum environmental conditions for cell separation (Merlo, 2003). The degree of cell separation in established cultures already having the characteristic of high friability can be modified by changing the composition of the nutrient medium. Increasing the auxin/cytokinin ratio will, in some cases, produce a more friable callus. On the other hand, some cultures exhibit low friability regardless of culture conditions (Scragg, 1992). There is no standard procedure that can be recommended for starting cell suspension cultures from callus; the choice of suitable conditions is largely determined by trial and error (Schemale *et al*., 2006).

The initiation of a cell suspension culture requires a relatively large amount of callus to serve as the inoculum; for example, ~2–3 g for 100 cm^3. When the plant material is first placed in the medium, there is an initial lag period prior to any sign of cell division. As with other cell culture system, the lag phase is followed by an exponential rise in cell number, and a steady increase in the cell population. There is a gradual deceleration in the division rate. Finally, the cells enter a stationary or nondividing stage. To maintain the viability of culture, the cells should be subcultured at the end of exponential phase or early during the stationary phase.

F. Transformation and transgenic cell line development

1. Agrobacterium

Transfer of a foreign gene into plant cells can be accomplished using a variety of methods. *Agrobacterium*-mediated transformation and particle bombardment are the two most widely used gene transfer methodologies. *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* have now been used for decades with many species exemplified. Initially, only certain dicotyledonous species were thought to be susceptible to infection by *Agrobacterium* (Chilton, 2001). However, later it was found that the *Agrobacterium* infection could be induced by adding certain supplements (Hiei *et al*., 1994) broadening greatly the number of plant species that can be used with the bacteria. Now, even monocotyledonous species such as grasses can be genetically engineered using *Agrobacterium* (Shrawat and Lorz, 2006).

2. Particle bombardment

Particle bombardment was developed in mid-to-late 1980s (Klein *et al*., 1988) and has been widely applied to numerous species. As usually practiced, small gold or tungsten particles are coated with plasmid DNA and the particles accelerated toward the target tissue using some device (Finer *et al*., 1992; Merlo, 2003). *Agrobacterium* usage results in transgenic events carrying just one copy of the transgene normally, while particle bombardment can introduce several to many copies of the transgene. This is important since multiple gene copies can result in gene silencing either initially or later in time.

3. Protoplasts, SiC fibers, and whole cell/direct gene transfer transformation

The use of plant protoplasts or SiC fiber-mediated transformation are less frequently used methods. The former method requires the production and purification of protoplasts following treatment of the plant tissue with fungal cellulases, hemicellulases, and pectinases. Plasmid DNA can then be introduced either via electroporation (Shillito *et al*., 1985) or using polyethylene glycol (Potrykus *et al*., 1985). Either method can introduce one or multiple gene copies. Moreover, the enzymes required can be costly, and sourcing them can be a problem. Whisker-mediated gene transfer is a relatively recent method and requires the use of silicon carbide (SiC) fibers. The plasmid DNA is coated onto the fibers and the tissue and coated fibers are placed into a small vessel that is agitated back and forth (Kaeppler *et al*., 1990). The SiC fibers puncture the cells delivering the DNA, which is sometimes incorporated into the host cell genome.

For the transformation of suspension cells, *Agrobacterium*-mediated transformation is routinely used. Rice cells can be directly transformed by cocultivation of suspension cells and *Agrobacteria* for 3–5 days prior to addition of antibiotics to eliminate the *Agrobacterium*. The selection of transgenic callus via hygromycin resistance requires 8–10 weeks and the reinitiation of suspension cell lines from transformed events can be achieved in less than 2 months. Other methods require approximately the same time (Merlo, 2003).

4. Transgenic cell line selection and characterization

It is well known that the establishment of a stable, high-expressing cell line is essential to enhanced production. Both recombinant protein production and cell growth are highly variable in plant cell culture. The successful scale-up of plant cell culture for the production of recombinant protein production in bioreactors is mainly dependent upon the optimal combination of three matrices: (1) transgenic cell line, (2) media components, and (3) bioreactor type and operation.

The selected transgenic cell line is the key player. Some of the most important attributes are:

- extremely high expression of the product;
- consistent expression with minimum variation to ensure predictability;
- high biomass accumulation, if it is positively correlated to the expression levels;
- minimum growth cycle to boost the number of batches per unit time.

a. Reculturability and growth profile Successful establishment of the transgenic cell lines depends upon the initial transgenic callus on the selection media. Transgenic cell line development begins with the choice of healthy and fast-growing callus events. The selected callus events are then bulked up for initiation of suspension cultures.

A kinetic analysis of cell growth, production formation, and nutrient consumption are very important for large-scale plant cell cultivation in bioreactors. In view of the fact that cells from different species vary in the length of time they remain viable during the stationary phase, it may be prudent to subculture during the period of progressive deceleration. Culture passage time and inoculum division and transfer can only be learnt from experience, and a given suspension culture should be subcultured at a time approximating the maximum cell density. For many suspension cultures, the maximum cell density is reached within about 18–25 days, although the passage time for some extremely active cultures may be as short as 6–9 days (Scragg, 1995). At the time of the first subculture, it will be necessary to filter the culture through a nylon net or stainless steel filter to remove the larger cell aggregates and residual

inoculum that would clog the orifice of a pipette. Biomass, growth rate, and product yields are three key factors that determine suitability.

b. Other approaches Numerous approaches have been taken in order to increase the yield of recombinant protein products in plant cell fermentation. These are:

- changes in medium composition (e.g., carbon source quantity and quality, nitrogen, phosphate, and growth regulators);
- changes in culture conditions (e.g., pH, temperature, pO_2, and light);
- specialized techniques (e.g., elicitation, and product removal).

G. Cell banking and cryopreservation

1. Cell banking: A three-tier cell banking system

To obtain a cell source that guarantees a constant and high level of biosafety and quality of the producer cell line, a cell-seeding system known as a tiered banking system or Master Cell Banking system should be established. A three-tier setup consisting of a research cell bank, a master cell bank (MCB), and a working cell bank (WCB) is generally accepted as the most practical approach for the establishment and validation of the producer cell line for both mammalian and plant cell lines; it has also been widely accepted as a standard (Schemale *et al.*, 2006).

On initial arrival into the laboratory, a new cell culture should be regarded as a potential source of contamination from bacteria and fungi, and should be handled under quarantine conditions until proven negative for such microbial contaminants. Following initial expansion, 3–5 ampoules should be frozen as a token stock before a Master Bank is prepared. One of the token stock ampoules should then be thawed and expanded to produce a Master Bank of 10–20 ampoules, depending upon the anticipated level of use.

Ampoules of this bank should be allocated for quality control, comprising confirmation that the cell count and viability of the bank is acceptable and that the bank is free of bacteria/fungi. Once these tests have been completed satisfactorily, an ampoule from the Master Bank should be thawed and cultured to produce a Working Bank. The size of this bank will again depend on the envisaged level of demand. Quality control tests (cell count and viability and the absence of microbial contaminants) are again performed prior to using the cultures for routine experimentation or production.

Implementation of this banking system ensures:

- material is of consistent quality;
- experiments are performed using cultures in the same range of passage numbers;

- cells are only in culture, when required;
- the original cell line characteristics are retained.

2. Documentation of history of the cell line

The clear and thorough documentation of the history of the cell line intended to be used for the production of a biopharmaceutical therapeutic is an important requirement for the establishment of the cell bank. This includes details on the origin of the cell line, as well as its passage history. The original tissue from which the cell line was taken and the method of isolation have to be stated. The documentation also has to include detailed information regarding the culture and storage conditions, and the media used for cultivation and cryopreservation. This intensive documentation effort has two main objectives: (1) to provide all the information needed to decide whether a cell line is safe enough to be used for the production of biopharmaceuticals; and (2) to provide all the data necessary to estimate the validation and testing effort needed to prove that the cell line satisfies the required biosafety regulations.

3. Qualification of cell line free of contamination

The cell line that is chosen for the manufacture of the biopharmaceutical must be proven to be free of contamination before the evaluated cells can be used for the preparation of a MCB, which contains the cryopreserved primary stocks of the cell line to be used. Cells from this stock are used to generate the working stock of cryopreserved cells called the manufacturer's working cell bank (MWCB) or just WCB. This stock serves as the only cell source for the production process. Cells taken from the WCB are used only for a particular production time. This has to be defined by careful studies to guarantee that production remains stable. Although it is customary for one vial to be used to generate a single batch, cells can be used for several production batches during the predetermined stable time span. When the end of the functional life is reached, cells are usually discarded and another vial is taken from the WCB to establish a new cell population that is used for subsequent production batches during the next span (Schemale *et al.*, 2006).

To prove the biosafety of the producer line, the MCB and WCB have to be validated. For this purpose, cells are taken from the cell bank and characterized with respect to their phenotype and genotype to document their identity and determine their genetic stability. The cells are also subject to tests for the presence of contaminating agents. These tests have to cover a wide range of possible contaminants such as bacteria and fungi, to ensure a high level of biosafety.

The establishment of cell banking systems, in combination with a detailed documentation of the cell line and careful validation procedures, reduce the risk of introducing contaminating agents via the cell line used

for production. It therefore provides a tool for manufacturers to maintain a high level of biosafety and high-quality standard of producer cell line during the entire life span of the biopharmaceutical product.

4. Benefits of cryopreservation

The aim of cryopreservation is to enable stocks of cells to be stored as a uniform, reproducible stock of genetic material. It is invaluable when dealing with cells of limited life span, a problem not usually associated with plant cells. It is improper practice to maintain a cell line in continuous or extended culture without a suitable long-term storage solution for the following reasons:

- risk of microbial contamination;
- loss of characteristics with culture age (e.g., surface antigen or monoclonal antibody expression);
- genetic drift, particularly in cells known to have an unstable karyotype;
- risk of cross contamination with other cell lines;
- increased consumables and personnel costs.

A generalized scheme for suspension cell line development to the point of cryopreservation is shown in Fig. 3.1. The various steps include transformation and selection, suspension cell line establishment, product study, and cryopreservation.

a. Cryoprotectants and general plant cell cryopreservation procedure

There has been a large amount of developmental work undertaken to ensure successful cryopreservation and resuscitation of a wide variety of cell lines of different cell types. The basic principle of successful cryopreservation is a slow freeze and a quick thaw. Although the precise requirement may vary with different cell lines, as a general guide, cells should be cooled at a rate of $-1\,^{\circ}C$ to $-3\,^{\circ}C$ per minute and thawed quickly by incubation in a $37\,^{\circ}C$ water bath for 3–5 min. If this and the additional points given below are followed, then most cell lines should be cryopreserved successfully.

Cultures should be healthy with a viability of $>90\%$ and no signs of microbial contamination. Cultures should be in log phase of growth at the time of freezing. Using a cryoprotectant such as dimethyl sulphoxide (DMSO) or glycerol helps to protect the cells from rupture by the formation of ice crystals. The most commonly used cryoprotectant is DMSO at a final concentration of $\sim 10\%$. However, this is not appropriate for all cell lines where DMSO is used to induce differentiation. In such cases, an alternative such as glycerol, sucrose, or mannitol may be used. These ingredients may also be combined in various proportions. Cryopreservation methods such as vitrification or other slow or staged freezing protocols are typically employed for plant cells.

b. Ultralow temperature storage of cell lines Cryopreservation has been successfully reported for rice, maize, and cotton in the literature. It is routine procedure to store the embryogenic suspension culture in liquid or vapor phase nitrogen. For vapor phase nitrogen storage, the ampoules are positioned above a shallow reservoir of liquid nitrogen, the depth of which has to be carefully maintained. A vertical temperature gradient will exist through the vapor phase, the extremes of which will depend on the liquid levels maintained, the design of the vessel, and the frequency with which it is opened. Temperature variations in the upper regions of a vapor phase storage vessel can be extreme if regular maintenance is not carried out.

All liquid nitrogen storage vessels should include alarms that at least warn of low liquid nitrogen levels. This is particularly true of storage systems used for the vapor phase. The bulk liquid nitrogen storage vessel should not become less than half before it is resupplied. This will ensure that at least one delivery can be missed without catastrophic consequences.

c. Assessment of postthaw recovery The presence of postthaw cell regrowth can be determined by regular (at 7-day intervals) examination of the thawed cells under a binocular microscope. Such examinations can be combined with an assessment of morphological features, such as color and wetness of the thawed cells, which can be important indicators of low temperature injury. Growth can be quantified by fresh and dry weight determinations, and growth expressed in terms of percentage of change after thawing or percentage difference as compared with nonfrozen controls. In combination with such observations, it is also useful to assess the viability of the thawed cells.

Viability determinations can provide a useful indication of potential postthaw growth and can be used to ascertain the effects of different postthaw culture conditions. However, such determinations should not be made in isolation, since apparently high cell viability soon after thawing is not always a prelude to postthaw cell growth.

d. Inventory control All ultralow-temperature storage vessels will include a racking/inventory system designed to organize the contents for ease of location and retrieval and supported by accurate record keeping and inventory control incorporating the following.

Each ampoule should be individually labeled, using ''wrap around'' liquid nitrogen resistant labels with identity, lot number, and date of freezing. The location of each ampoule should be recorded ideally on an electronic database or spreadsheet, but also on a paper storage plan. There should be a control system to ensure that no ampoule can be deposited or withdrawn without updating the records. Two-dimensional bar code labels can be circular in nature and used for sample tracking within the cryotank.

H. Quality control considerations on plant cell fermentation

Quality is important in all aspects of tissue culture since the quality of materials used (i.e., media and other reagents) will affect the quality of the cultures and products derived from them. The main areas of quality control that are of concern for tissue culture are:

- the quality of the reagents and raw materials;
- the provenance and integrity of the cell lines;
- the avoidance of microbial contamination.

1. Reagents and raw materials

A potential source of contamination is through reagents and raw materials. Good quality reagents and materials are available from numerous manufacturers of tissue culture media and supplements. In addition, manufacturers will carry out a range of quality control tests including screening for microorganisms and supply a Certificate of Analysis (CoA) with their products. These state the product and lot numbers and form a vital part of record keeping and tracking of reagents used in the production of cell stocks. It is advisable to further test key reagents to ensure that they are ''fit for purpose'' despite batch-to-batch variation.

Manufacturers of sterile plasticware (flasks, centrifuges tubes, pipettes) designed for tissue culture use are also supplied with a CoA for each batch produced, which should be kept for future reference.

a. Culture media and raw materials The composition of the culture medium is the most significant factor for cell culture initiation and cell culture growth. Cells can deteriorate and disintegrate (lyse) when nutrient supply is inadequate.

Different kinds of cells require different media, and vendors offer preformulated media designed for all of the cells widely used in bioprocessing. In addition, to the nutritive elements, media sometimes contain additives designed to improve the fermentation process. Pluronic F68, for instance, is used to make cell membranes more resistant to shear forces. Polyethylene glycol, silicone-based antifoams, or other agents are used to reduce foaming.

Although the term ''raw material'' is widely used in the literature, it is not always clearly defined. Here, raw materials (RMs) are defined as the chemical, biochemical, or biological components that are used in a process to manufacture a biopharmaceutical product. Although the cell material used for the cultivation process is often considered to be a raw material, it is excluded from the definition because there are special aspects to consider regarding cells.

During the course of a production process, RMs are used for many different purposes. They are needed, for example, for the cultivation of cells, the isolation and purification of the desired product, or as excipients for the final product formulation, as well as for the cleaning and maintenance of production equipment. Therefore, different categories of RMs can be distinguished, reflecting their use or purpose during the process and their presence in the final product.

In light of the definition given above, RMs includes all substances that are introduced into the manufacturing process of a biopharmaceutical product. Therefore, RM is the most critical source of possible impurities or contamination, with the exception of the producer cells. However, not all RMs contribute to this risk to the same extent. According to their categories, different safety and quality standards have to be applied to RMs. Components that are present in the final production formulation, for example, must comply with pharmacopoeial and regulatory standards, although manufacturer-defined standards can be applied to RMs that are used for cleaning purposes. The fate of a given RM throughout the process, with respect to its contact with the product components or its presence in the final formulation, should be considered in this context. As a result of these considerations, a procedure for the quality and safety validation of RMs used for manufacturing biopharmaceuticals should be designed.

Two strategies are used to ensure the quality and safety of RMs. First, an RM should be tested to verify its identity, purity, and safety, as well as its suitability for the process. However, this can be difficult to achieve for complex RMs if they have a biological origin. The second strategy is clear determination and validation of the origin and source of the RMs and is of similar importance to the first strategy.

Tracking RMs from their origin not only assists in verifying the identity and purity of RMs, but also enables the characterization of the most likely contaminants. This is of great value when deciding which biosafety tests should be included in the testing scheme. Knowing the history of production and the source of RMs is especially important for complex RMs of biological origin because contaminating agents might be present that are unknown or cannot be detected easily. In these cases, validation of origin, manufacturers, and vendors of RMs is the only way to guarantee a certain degree of biosafety. The problem can only be overcome by the establishment of a functional vendor-audit program.

2. Provenance and integrity of cell lines

The sourcing of cell lines can have an important effect on quality, since freshly imported cell lines are a major source of contamination. The

advantages of obtaining cell lines from a recognized source such as a culture collection are:

- contaminant free;
- fully characterized and authenticated in terms of DNA profile and species of origin;
- supplied with a detailed data sheet.

Once the cell lines have been obtained from a reputable source, it is important to implement master and working cell banking procedures and the associated quality control steps such as routine testing for microbial contaminants and confirming the identity of cultures.

3. Avoidance of microbial contamination

Potential sources of contamination include other cell lines, laboratory conditions, and staff poorly trained in core areas such as aseptic techniques and good laboratory practice. Thus, the use of cells and reagents of known origin and quality alone is not sufficient to guarantee quality of product (cell stock or culture products); it is necessary to demonstrate quality throughout the production process and also in the final product. Routine screening aids the early detection of contamination, since all manipulations are a potential source of contamination. Bacterial contamination is generally visible to the naked eye and detected by a sudden increase in turbidity and color change in the culture medium resulting from a change in pH. The cell culture may survive for a short time but the cells will eventually die. Daily microscopic observation of cultures will ensure early detection of contamination and enable appropriate action to be taken as soon as the first signs of contamination become apparent (explained later). In addition, specific tests for the detection of bacteria and fungi contamination should be used as part of a routine and regular quality control screening procedure.

4. Authentication of cell lines

Whatever the scope of work to be carried out, it is important to know that the work is being conducted using the correct reagents. This is no less important for cell cultures, since if the cell cultures are not what they are reported to be, then the work can be invalidated and resources wasted. To minimize the risk of working with contaminated cell lines, it is advisable to obtain cells from a recognized source such as a culture collection that will have confirmed the identity of the cells as part of the banking process. Tests used to authenticate cell cultures include isozyme analysis, karyotyping and/or cytogenic analysis, and more recently, molecular techniques of DNA profiling. While most of the techniques above are generalized tests and are applicable to all cell lines, additional specific tests may also be required to confirm the presence of a product or antigen of interest.

III. INDUSTRIAL-SCALE PRODUCTION WITH PLANT SUSPENSION CELL CULTURES

Plant cell culture is a unique enabling technology that can be employed for the production of various biologically active compounds including podophyllotoxin (Chattopadhyay *et al.*, 2002a,b) as well as the expression of recombinant proteins and biopharmaceuticals (Doran, 1999; Fischer *et al.*, 2004; Ma *et al.*, 2003). Recent advances in plant cell culture have enabled the production of medicinally high-value secondary metabolites such as paclitaxel and related taxanes on an industrial scale in large fermentors, using suspension cell cultures (Dong and Zhong, 2002; Srinivasan *et al.*, 1995). This innovation comprising the use of plant cells in contained environment offers several benefits, such as improved production control, flexibility to meet production and scheduling needs, and enabling regulatory compliance for cGMP operations. Certain metabolites such as the naphthoquinone pigment shikonin, the alkaloids berberine and sanguinarine, and a few other products have been produced at multiple grams per liter (up to 25% dry weight) yield, for example, Ulbrich *et al.* (1985) reported the production of rosmarinic acid at 21% dry weight using *Coleus blumei* cultures and arbutin production up to 9.2 g/liter corresponding to 45% dry weight was obtained through feeding hydroquinone (Yokoyama and Yanagi, 1991) and paclitaxel has been produced at more than 5% dry weight (Bringi *et al.*, 1997; PTC/US97/08907) typical yields from plant cell cultures have been low (0.01–1%). Thus, depending on market demand, commercial production of phytochemicals or recombinant therapeutic proteins derived from culturing plant suspension cells (Hogue *et al.*, 1990) will require medium for large-scale fermentation processes (10–100 kl). In addition, it will require the development of superior and improved cell lines with certain desirable features (Table 3.4), which include fast growth rate, ease of genetic manipulation, culture stability, protein expression capability, low content of phytochemicals, and phenolics that may interfere with downstream processing and process optimization to increase the productivity several fold (James and Lee, 2001). The most widely reported host species used for developing recombinant products is tobacco (*Nicotiana tabacum*) followed by rice (*Oryza sativa*). Recent advances in bioinformatics, computer and interfacing of modern automation, and process control, along with the experience of large-scale production with submerged fungi fermentation by pharmaceutical industries, suggests that large-scale process development with other plant suspension cell lines may indeed be feasible. The following section addresses the various challenges on scaling-up (Table 3.5) and multiple strategies aimed at improving yields through synergistic interaction in bioreactor process development and optimizations relevant for large-scale operation with plant suspension cell lines.

A. Scale-up issues with plant suspension cell cultures

Significant process development efforts, before scale-up is initiated, are first performed in laboratory equipment identifying components that stimulate productivity. However, scale-up problems are commonly encountered with plant cells than with successful track history of microbial cells. The limitations and challenges of plant cell fermentation are: (1) lack of knowledge about the growth of plant cells and the expression of recombinant proteins in bioreactors, thus much more intensive research for bioreactors and the effective process development are needed; (2) poor growth rates and long doubling time, which means that plant cells produce relatively low biomass; (3) formation of large aggregates *in vitro*, thus more vigorous stirring is often needed to prevent this clumping, which, however, may damage the cells (Chattopadhyay *et al*., 2002a,b). Some of these problems have been addressed by improved bioreactor design, optimized agitation conditions, and nutrient supply optimization (Scragg *et al*., 1987). Other aspects depend on the plant species used and on a careful selection of the cell line with respect to product formation, growth characteristics, and genetic stability.

As with the classical ''antibiotic industry,'' there are some ''thumb rules,'' trial and error approaches, models, and other kinds of engineering science derived to successfully produce compounds from suspension cell culture. Here, a number of laboratory-scale experiments are performed to yield essential data, like kinetics of growth rate, product formation rate, nutrient uptake rate, respiration, heat, and mixing characteristics on scale-up from pilot to industrial conditions (Scragg, 1998; Tale and Pyne, 1991). However, this data is interpreted and mimicked with caution, because the overall performance yields and environmental and cultural condition in laboratory shake flasks and pilot and large tanks are vastly different. In most cases, empirical approaches, such as power draws to volume ratio, nutrient addition, oxygen transfer rate, impeller tip velocity, oxygen tension, and dissolved carbon dioxide (CO_2) level, and its disengagement from the suspension, are the parameters measured during process optimization and scale-up (Smart and Fowler, 1981). In such cases, newer and more sophisticated design concepts are implemented in order to culture plant cells. However, emphasis should be placed on using minimum engineering redesign and focusing on the ability to flexibly operate in the existing facility, thereby adopting the plant cells to the reactors, rather than introducing modifications of design to the facility to accommodate plant cell culture.

A large number of species of various plant cells in suspension have been successfully cultivated (Table 3.6) in various bioreactors (Buitelaar and Tramper, 1992; Gao and Lee, 1992; Taticek *et al*., 1994). While some plant cells form fine suspension with few aggregates, on an average less

TABLE 3.6 Various bioreactor modified and tested for mixing and optimal industrial culturing of plant suspension cells

Reactor design	Modification
Stirred tank (STR) with impeller and sparger	Flat blade turbine
	Large paddle
	Marine propeller
	Helical stirrer
	Paddle with spiral sparger
	Sintered filter sparger
	Pitch blades
	Anchor impeller
STR-bubble free	Surface aeration, surface baffles and helical ribbon impellers
	Membrane stirrers
Column reactors	Bubble column (airlift)
	Internal/external loop
	Draft tube
Perfusion	Spin filer reactor
	STR with cell retention filter
	Filtration bubble column
Rotating drum (RD)	Bubble aeration
	Surface aeration

than 1 mm in diameter or <25 cells/aggregate, such as with *N. tabacum*, others such as *Panax ginseng*, form large aggregates up to 2 cm in diameter. Formation of aggregates is mainly due to the cells not separating after cell division. Depending on the cell lines used (Table 3.7), conventional bioreactors such as stirred tank reactors (STR), airlift, and bubble columns have been used for plant suspension cultivation. As an example, *N. tabacum* suspension cells have been cultivated commercially in 20,000 liter STR and certain *Taxus* lines scaled-up to 75,000 liter (Srinivasan *et al.*, 1995) (Table 3.7).

B. Process optimization

This section is a review of variables and environmental factors that influence productivity, and the performance of plant cells on scale-up. The topics discussed here are broad and generalized, because the use of plant cell lines for process development are dependent on the cell line employed and the final product of interest. Other factors that should be considered are medium components and, in some cell cultures, the use of

TABLE 3.7 Examples of industrial plant suspension cell lines scaled-up beyond shake flasks

Cell lines	Reactor configuration	Volume (liter)
T. chinensis	STR	75,000
N. tabacum	STR	10–20,000
L. erythrorizom	Rotating drum	2–1000
C. Roseus	AL	100
C. Roseus	STR	14–5000
P. ginseng	STR	30–20,000
Echinacea purpurea	STR	75,000
Thalictrum rugosum	Membrane based	2
Solanum demissum	STR	800
Digilatis lanta	AL	200
Glycine max	STR	200
Helianthus annuus	AL	80

STR—stirred tank reactor
AL—airlift

substrate influences the product and growth formation. In certain other cases, elicitors, growth regulators, metal or inorganic ions, vitamins, and inducers are supplemented in the medium during growth for product formation (Srinivasan *et al.*, 1996; Taticek *et al.*, 1994).

1. Nutrients

The use of sugars and osmotic agents in suspension cultures has been focused on improving the productivity of secondary metabolites. Depending on the cell line, in most instances, carbon to nitrogen ratio and the supply of carbon sources have been recognized as important factors for cultivating the suspension cells. Several carbon sources such as glucose, fructose, mannitol, lactose, and maltose have been used. Greater success has been achieved with glucose or sucrose (Dong and Zhong, 2002; Martinez and Park, 1993; Srinivasan *et al.*, 1996).

Under osmotic pressure, plant cell physiology changes drastically. Osmotic stress mimics drought stress, and can induce secondary metabolism in plant cells or foreign protein expression by genetically engineered cells. There is limited data on the role of signal transduction system and the maintenance of osmolarity using osmotic stabilizing agents post induction on product formation. In some other instances, such as in paclitaxel, using a mixture of osmotic stabilizing agents like sucrose or nonmetabolic sugars such as mannitol or nonsugar osmotic agents such as polyethylene glycol (PEG) enhance productivity. Most of the osmotic

agents used have been noticed to have some role in maintaining the storage carbohydrates or intracellular metabolites. The stimulatory effect of conditioned medium addition on the production of physiologically active taxyunnainne C (Tic) has also been demonstrated. Among the manipulative techniques available to enhance the productivity of secondary metabolites from plant cell cultures, use of elicitors has been the most successful in markedly improving the product yield (Stafford *et al.*, 1986). Elicitors can be biotic, usually glucan polymers or fungal cell material, or abiotic, including UV, salts, methyl jasmonate, and heavy metals (Payne *et al.*, 1992).

2. Biomass

Of the various strategies optimized on scale-up, the one that supports maximum biomass in suspension culture has been the most important parameter considered, especially if the product is cell associated in vacuoles or cell compartments. Here various environmental conditions such as dissolved oxygen, carbon dioxide concentration in the broth, and other gaseous metabolites, as well as nutrient feed for optimum biomass, are studied (Srinivasan *et al.*, 1995). In fact, many industrial-scale problems and challenges, as those faced in mammalian cell culture, are also noticed with plant suspension cell culturing. These issues have resulted in the evolution and modification of several bioreactors and design configurations (Table 3.6) to manage cultivation of plant suspension cells (Doran, 2000). Factors that influence development of a process from plant suspension cells are the choice of bioreactors, the operation mode (batch, fed-batch, semicontinuous, draw, and fill), inoculation, stirring, and sparging. In a large stirred tank bioreactor (Fig. 3.2), culturing a semiheterogeneous plant cell mass, maintaining a uniform transfer of oxygen and carbon dioxide between gas and liquid phase, and establishing homogeneous distribution of nutrients and cells are difficult. While approaches have been evaluated to manage these parameters and provide optimal environment for other systems, with plant cell cultures, the application of various technologies to successfully scale-up processes are restricted by the physical, biological, and mechanical constraints. Hydrodynamic stressing, turbulent flow, and vigorous agitation and aeration in some shear-sensitive cell lines damage the cells and result in poor performance (Scragg *et al.*, 1988). Furthermore, the rheology of the liquid during the course of large-scale operations also changes (Curtis and Emery, 1993; Scragg, 1995). For example, with most plant suspension cells, at a high biomass concentration and at the late stage of growth, an increase in cell size coincides with the excretion of extracellular polysaccharides, thus making the culture extremely viscous. The scale-up strategy in such cases has been to find the right balance between aeration, agitation, gas composition, and controlling hydrodynamic force (Curtis

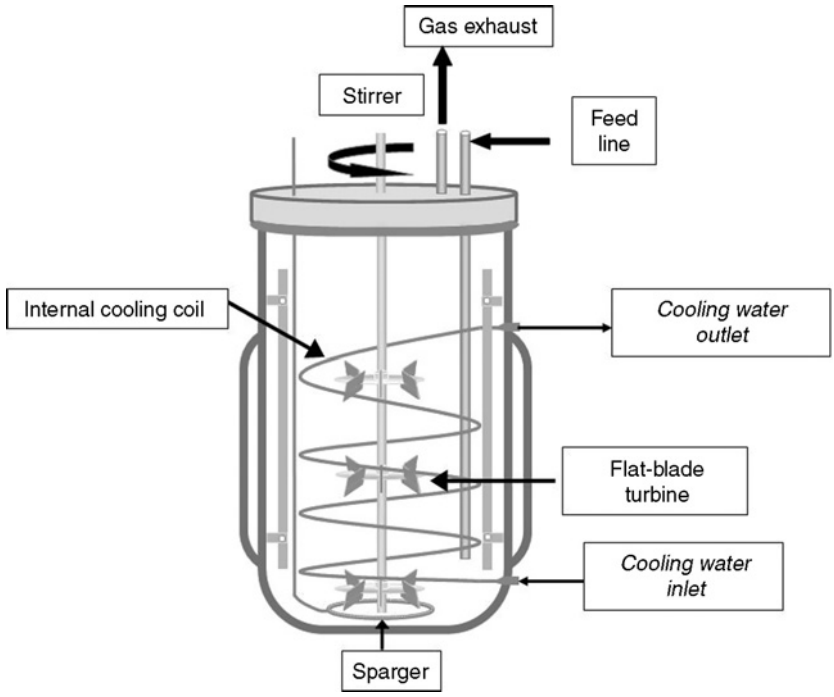

FIGURE 3.2 Typical STR.

and Emery, 1993). Several types of exotic bioreactors have been designed for specific plant cells to achieve desired high cell density and a right balance of product expression (Hooker and Lee, 1992). However, stirred tanks are most widely and often used. Most of these reactors, which include the rotating drum, roller bottle, annular vortex membrane reactor, centrifugal impeller bioreactor, and balloon type bubble reactors (Table 3.7), have not proceeded beyond academic curiosity or scaled-up for pilot demonstration up to volumes of 100–500 liters (Doran, 1999; Scragg, 1998). Doran (2000) concluded that for high-density plant cell cultures (300 g/liter fresh weight), mixing becomes a limiting factor in airlift fermentors. Another problem with airlift and pneumatically agitated reactors such as bubble columns is foaming. Airlift and bubble column reactors work well at low cell densities (Figs. 3.3 and 3.4).

However, to increase reactor volumetric productivity, it is generally preferred to operate bioreactors at high cell densities and thus STR remains the reactor of choice. An integral part of designing the STR for culturing plant cells requires an understanding of how to set and manage the operating conditions such as aeration, heating, cooling, agitator speed so that appropriate oxygen demand can be met to improve product yield

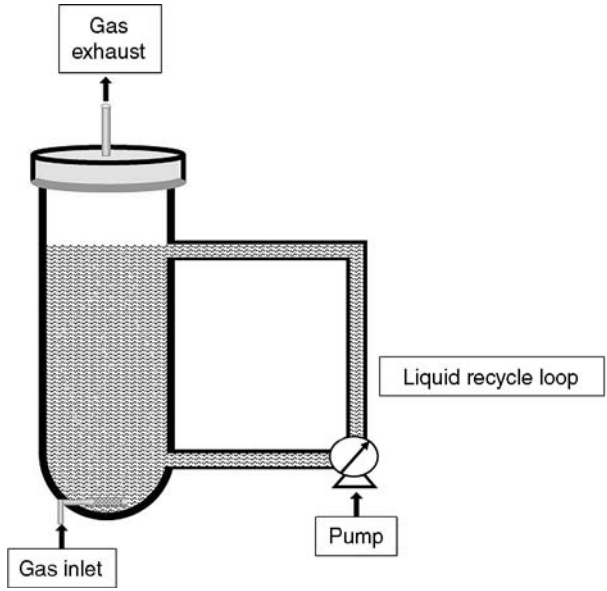

FIGURE 3.3 Bubble column reactor with external circulation.

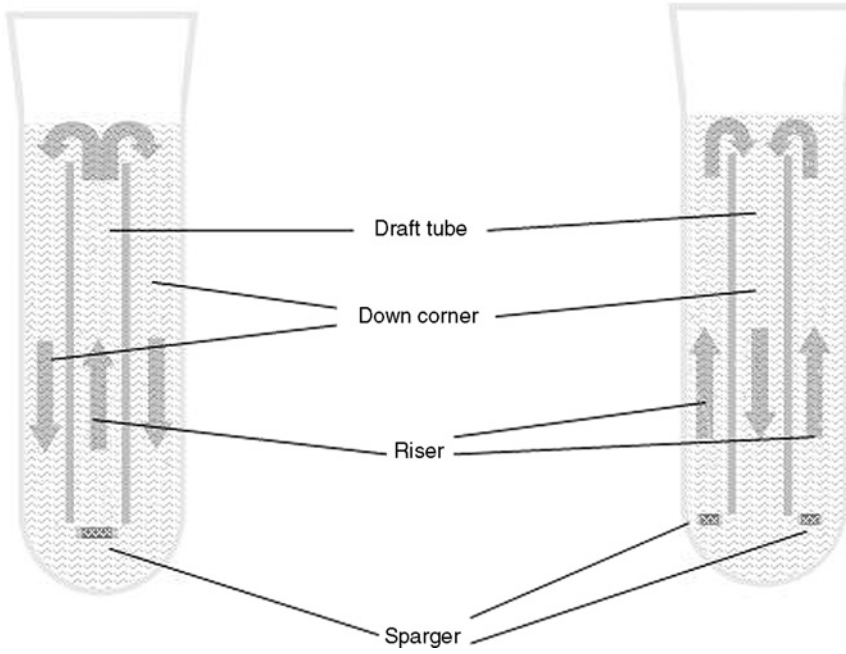

FIGURE 3.4 Bubble column reactor with drafts.

without causing excessive shear damage and foaming. Airlift reactors are commonly used for shear-sensitive plant cells.

In some plant cell cultures, biomass from 30 to 137 g/liter has been reported (Park and Kim, 1993). To attain a higher level of growth and biomass on a larger scale depends on the optimum supply of nutrients, substrate feed, and water content of cells. In practice, fresh weights over 200 g/liter are possible on a commercial scale with either airlift or STR with a fed-batch process. However, with the higher cell densities, one should have the ability to successfully manage the transfer of nutrient and the supply of substrate and oxygen for optimal product yield as these are important process optimization components (Gao and Lee, 1992; Tale and Pyne, 1991).

3. Inoculum

Minimum inoculum is essential for optimum growth of plant cells and product expression. In most instances, a minimum of 0.5% (wet weight/volume) serves the purpose. The reason for this minimum level is not clear, but several studies on inoculum have suggested the presence of a stimulatory metabolite in spent media. Substituting spent media with water or fresh media have resulted in longer lag phase, lower growth rate, and heterogeneous population. Some investigators indicate the presence of a key metabolite may be critical, and seeding with minimum inoculum cell density serves this purpose. Besides the inoculum, the physiological stage of the inoculum is also critical for the batch process (Srinivasan *et al.*, 1996). Typically, for recombinant protein production, a plant cell culture with a fast growth rate is preferred. It is for these reasons that tobacco BY-2 and rice lines are particularly appealing because of their rapid growth rate and ease of recovering recombinant strains. Doubling times, as short as 11 h, for young dividing cells have been reported. In addition, for growth-associated production (e.g., alkaloids), it has been noticed to be higher with juvenile cultures. This variable must be addressed with caution, especially in certain two-stage processes with plant cells, since repeated use of young dividing culture in a bioreactor may lead to progressive selection of fast-growing cells in place of the desired, high producing slow growing cells.

4. Gas Mass transfer and shear

Optimum cultivation of plant cells expressing recombinant proteins in any bioreactor requires efficient supply and distribution of nutrients, oxygen transfer as well as removal of undesired volatile and spent gases. Oxygen, carbon dioxide, ethylene are all important in plant cell system. Ethylene is a gas component produced by plants as kind of protection response under provocation of stressful environment. Air-driven bioreactors are solely dependent on aeration for transport of

nutrients and mixing (Fig. 3.3 and 3.4). These bioreactors are not the first choice for high cell density culturing because of their suboptimum mixing characteristics, causing some anaerobic zones and heterogeneous mixing and low cell mass formation (Scragg, 1995). However, internal loops with high air velocities have been applied to get better axial distribution. The use of high air superficial velocity in most instances has resulted in high shear and low productivity (Tanaka, 1981). In some studies, dissolved oxygen levels have been found to be critical at all the stages of biomass, product formation, and maintenance. Furthermore, in some plant cell cultures where the rheology of the media alters with the progress of the batch, a sufficient level of oxygen needs to be pumped in order to maintain viability and product expression (Taticek *et al.*, 1991). The critical level of dissolved oxygen and control and percent saturation are cell line, product, and process dependent. The critical level of dissolved oxygen in plant cell culture is typically at 15–20% air saturation. In others, tolerance to low oxygen tension is expected to be species dependent especially for those cultures when operated at high cell density in large-scale bioreactors. This requires bioreactor design modification to combat oxygen supply problems. For example, in most cases, insufficient removal of carbon dioxide from the broth has been detrimental to certain cell culture (Scragg *et al.*, 1988). However, there are no fixed rules, because in the case of *Cathranthus roseus*, ajmilicine production improved with carbon dioxide tension in the media (Drapeau *et al.*, 1987). Literature reports on dissolved oxygen and carbon dioxide levels have been inconsistent and variable (Scragg, 1995; Tale and Pyne, 1991). Depending on the culture conditions, interaction between CO_2 and ethylene, and between ethylene and methyl jasmonate could significantly influence production of secondary metabolite such as paclitaxel. This specific variable is assessed on a cell line-to-cell line and case-by-case basis (Srinivasan *et al.*, 1996).

In a STR, mixing and nutrient transfer are achieved with various impeller types and configurations (Fig. 3.5). Most reports in cell suspension show that only the power input or energy dissipation in the vessel determines the mass transfer (Curtis and Emery, 1993; Hooker *et al.*, 1990; Scragg, 1995). However, rapid mixing time and resident time distributions of nutrients need to be modeled for specific plant cell lines simultaneously taking into consideration the specific configuration employed in the STR. Typically, efficient mixing and mass transfer is achieved by increasing the impeller speed. Though the mixing pattern is important during scale-up of plant cells, it plays a minor role. Most studies indicate that airflow, impeller configuration, speed and the hydrodynamic shear generated, bubbles, and foam are critical during cell culturing.

Because, plant cells are considered intrinsically shear sensitive, there are speculative negative reports on using STR for large-scale practice and recommend against using them in production systems (Namdev and

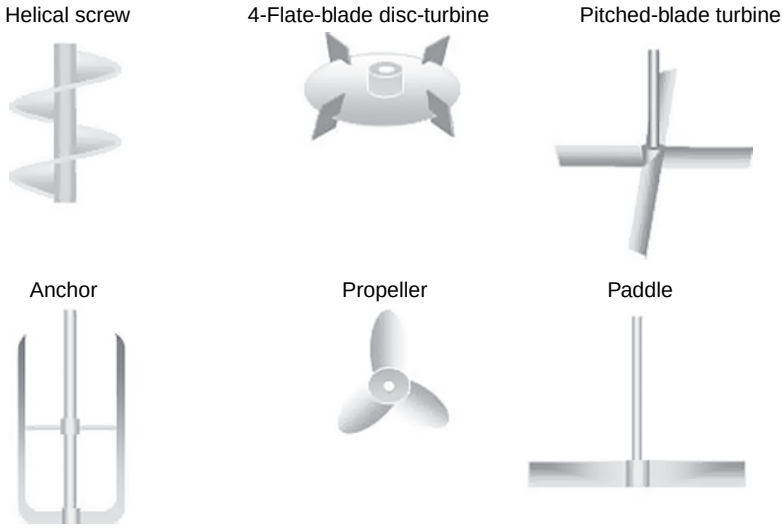

FIGURE 3.5 Various impeller types used for efficient mixing in STR.

Dunlop, 1995; Tanaka, 1981). However, most recent studies performed on various cell lines have shown that plant cell species are in fact shear tolerant (Scragg *et al*., 1988). More often with certain plant cell suspensions, the cell is routinely cultured and maintained in the liquid media for a long time and then transferred, so the cell becomes acclimated to the liquid environment. This data was supported by the fact that cultivating a freshly initiated calli of *Ginseng* culture on a large-scale presented culturing problems due to shear effects (Scragg, 1998), while the cultivation of *Taxus chinensis* cell lines maintained in the liquid media were successfully scaled-up to 75 kl (Fig. 3.6 and 3.7) and presented no problem (Robert and Schuler, 1997). In general, most reports on successful cultivation of plant cells at large-scale indicate them to not be sensitive, as a rule. In several instances, options for overcoming the negative effects of shear imposed by impellers, foam, and bubble coalescence have been to modify the impeller location, size, and configuration (Table VI). In other instances, replacing the common Rushton turbine impellers with hollow paddles, the marine, the cell lift, large flat blade, helical ribbon type impellers seem to have led to better performances with the plant suspension cells (Hooker *et al*., 1990).

During process scale-up, the size and the geometry of tanks are altered and so one must consider and take into account these changes, because these changes also result in increased hydrostatic pressure between the top and the bottom of the vessel. How this affects mixing and plant cell physiology is not certain. However, based on large-scale microbial operations, such modifications have influenced the solubility of gas (Scragg,

FIGURE 3.6 cGMP bioreactor cascade for plant cell suspension-based therapeutics production at Phyton Biotech.

FIGURE 3.7 View of the 75,000-liter bioreactor at Phyton Biotech's manufacturing facility.

1995; Tanaka, 1987). For example, raising the back pressure or overhead pressure of the vessel (above the liquid space in tanks), typically results in increased mass transport of oxygen from the gas phase to bulk fluid and, consequently, facilitates the amount of bubbles (gas hold-up) in the large tank. While this effect has been supportive for plant cells consuming oxygen at peak demand, this change also necessitates the need to disengage the dissolved carbon dioxide produced by actively respiring plant cells from the broth. The effect of CO_2 is more pronounced in tall and

large bubble column reactors, since rheological properties of broth and cells also dictate the efficiency of gas removal (Tale and Pyne, 1991).

5. Foam

Foaming can be expected in aerated suspension plant cell cultures. A number of factors are believed to contribute to foam formation. These include presence of extracellular polysaccharides, proteinacious substrate, fatty acids (secreted by lysed cells), and high sugar concentration during early stages of growth. The extent of foaming is affected by condition of operations namely aeration rate, media composition, broth viscosity, biomass level, and internal configuration of bioreactors. A special feature of many plant suspension cells culturing in large tanks, is their tendency to get entrapped in the foam layer, and reside in this foam layer, thereby creating a solid meringue above the culture liquid (Abdulla *et al*., 2000). It is clear that such foam-related biomass is undesirable, and must be avoided as this can reduce the biomass level in the bulk liquid. The cells in this meringue are subjected to nutrient and oxygen deficiencies and may lead to premature cell death. Leakage of cell content into the culture also leads to undesired side effects making it difficult to scale-up the process. In some instances, accumulated cells form a thick crust adhering to vessels and probes. Under severe foaming, foam overflow can clog the air vent filters and can make the batch susceptible to contamination. In extreme conditions, foam formation and wall growth have led to an empty vessel within hours after foam formation (Scragg, 1995). Therefore, foam control is an important aspect of process optimization during scale-up of plant cultures. The need to avoid the formation of a stable foam layer on the large tanks is well documented (Abdulla *et al*., 2002). Reduced aeration rate, use of mechanical foam breakers, silicone oil, and other surface active agents all have been suggested to repress foam formation (Kawase and Moo-Young, 1990). In other instances, using an impeller installed above culture broth surface to serve as mechanical foam breaker has met with limited success (Su, 2006). Fortunately, as the reactor is geometrically scaled-up, the reactor cross-section per volume ratio drops and the wall growth and other foam problems are expected to reduce. There are no reports on the negative effects of using antifoaming agents in plant cell suspensions (Doran, 2000).

6. Temperature and light

Temperature, light control, and removal of heat are also important parameters in industrial plant cell culturing, but have received little attention (Morris, 1986). The temperature range for the growth of plant cells is between 15 and 32 °C. Generally, the optimal temperatures range between 23 and 30 °C and are species dependent. At temperatures above 35 °C, the growth rate decreases, perhaps due to inactivation of

intracellular enzymes and cell damage. Lower temperature results in suboptimum growth and in a few cases; cell growth is inhibited. Most large-scale microbial cell fermentations are exothermic, with heat generated due to growth, microbial activity, and power input by aeration and agitation. In most instances, circulating cooling water through internal coils or water jackets removes the heat generated in the broth. In the case of plant suspension cells, the heat accumulation is primarily caused by power input of motor and not overall metabolic activity, which is low because of slow growth. Since there is an inverse relation between the temperature and solubility of gasses, operation of plant cells at ambient temperatures enables management of critical dissolved oxygen and oxygen transfer rates (Park and Kim, 1993).

There is limited data on the effect of light on the productivity of plant cell culture. Light, in certain cell lines, seemed to influence the formation of unwanted pigments, secondary metabolites and compounds. This is not a critical component during scale-up, since most large tanks over 10 liters are stainless steel and have limited access to light. However, the formation of unwanted pigments, secondary metabolites and compounds is important in cases where the cell line stability and product formation is light dependent.

C. Product formation

Product formation (yield) in plant cell suspension is dependent on process conditions, the cell line, and its capability to translate the product (intra- or extracellular) during biosynthesis. The production occurs either predominantly during active cell growth (i.e., growth associated) or after active cell growth has ceased (i.e., nongrowth associated). In general, the expression of recombinant protein is driven by the type of promoter used. When a constitutive promoter such as the cauliflower mosaic virus (CaMV) 35S promoter is used to drive transgene expression, protein production occurs during growth phase. Hence, the factors that promote growth, such as improved DO supply, are expected to promote protein expression. If an inducible promoter is used, the transgenic expression is induced after the culture reaches high cell density, mostly during the late exponential phase (Trexter *et al*., 2002). In this case, the production is decoupled from growth. In order to maximize the efficiency of inducible gene expression, it is necessary to test the inducer concentration and time of induction. For the production of certain secondary metabolites, however, a two-stage batch process, a growth phase followed by product formation stage (Misawa, 1985), is recommended. In such a process, an effort at production stage is first directed toward minimizing the flow of raw materials through the metabolic pathways toward biomass formation and redirected towards maximizing product

formation. However, there can be instances where product of interest and biomass are sometimes inversely related (Noguchi *et al*., 1987). No matter how the final production is managed, the two critical process variables that dictate optimum product formation on scale-up are: biomass concentration C_x (g/liter, i.e., grams of biomass per liter) and the specific production rate Q_p (kg/g/h, i.e., kilograms of product per gram of biomass per hour).

$$R_p = Q_p \times C_x$$

Productivity in plant cell culture is typically determined by R_p, measured in kg of product synthesized in liters of volume/hour and are driven by three factors: $R_p = Q_p \times C_x$, where C_x represents the biomass concentrationmg/liter and Q_p is product formation rate (time dependent).

The specific production rate is the most critical process optimization factor (Su, 2006). This variable is dependent on C_x (active biomass that drives product formation). Essentially, Q_p relates to the component of active biomass engaged in producing the final product and not the overall dry weight. In large-scale operations, Q_p or specific rate of product is governed by two elements $Q_p = C_x \times \text{Max}\, f$ (environmental factors). The specific productivity, Q_p, is a biological parameter—driven by the parameter encoded in the genes of the cell line. Srinivasan *et al*. (1996) proposed a strategy to optimize this component including the selection and development of elite cell lines having the ability to over express genes encoded for rate limiting enzymes. In addition, raising copy number, increasing the half-life of critical enzymes, and inhibiting enzymes competing for critical metabolic pathway, or stabilizing and extending the product formation step have also been noticed to have a positive impact (Buitelaar and Tramper, 1992). Since these parameters are influenced by environmental factors, optimization typically focuses on those factors that may have an effect on gene expression, enzyme activities, and cell viability during scale-up (Srinivasan *et al*., 1996). A variety of molecular strategies exist for improvement of gene expression and heterologous protein accumulation in plant cells. Included among these are the use of appropriate promoters, enhancers, leader sequences, optimization of codon usage, control of gene copy number, and subcellular targeting of gene products (e.g., by using an ER-targeting signal peptide). In some other cases, nuclear matrix attachment regions (MARs) have been found to increase transgene expression (Spiker and Thompson, 1996). Thus, during optimization, emphasis is focused typically on minimizing negative effects of environment and accelerating cell vitality, biomass, product expression, and ultimately maximizing Q_p.

D. Process operation strategies on scale-up

Based on the literature and experiences in plant cell culture, Drapeau *et al.*, (1987) stated that the economic feasibility of culturing suspension plant cells is primarily determined by the system productivity. For those products that are intracellular in plant cells, it is governed by biomass level C_x, and product formation rate, Q_p. Production of plant cell biomass, where product is cell associated can be performed in batch or fed-batch mode. Fed-batch operations facilitate the change from growth to production medium without separation of spent growth medium and biomass.

In certain processes, high-density cell cultivation is generally considered a useful technique to enhance yield and productivity, as found with various plant cell cultures in recent years. In cell culture of tobacco, Su (2006) conducted high-density cultivation by varying the initial sugar concentration in the medium. The results indicate that the success of plant cell scale-up for the production of recombinant proteins is mainly dependent on the quality of the transgenic cell line used. This quality is established and maintained by ongoing programs of the initiation of transgenic cell lines from callus events, screening, and selection.

Economic feasibility is also process and operation driven (Su, 2006). For example, a two-stage process in which the first stage uses the cell growth medium, biomass is produced at the highest rate before it reaches stationary growth phase, and in the second stage, the production media is supplemented with the elicitors designed to allow for product to accumulate at a high rate. In such cases, it is essential to determine total bioreactor volume for specifying production. Carrying the production in more than one reactor also splits the risk of losing the batch to contamination. If the second stage (production) is longer than the first stage (biomass growth or seed), a more economical use of bioreactor train for biomass growth is possible by feeding more than one production fermentors from one seed tank. For example, if it takes 5 days to grow the seed for production tank and the resident time in the production stage is 10 days, then it is economical to use the first stage seed tank to seed two production tanks simultaneously.

The use of a two-stage process has proven to be the most successful for the production of secondary metabolite. Many recombinant proteins produced are at least partially growth associated and therefore the link between growth and production needs to be carefully examined (Doran, 2006). The use of inducible systems such as the α-amylase promoter, which responds to sugar starvation, can be used to create a clear separation between growth and product accumulation phases. However, strategies involving post-growth phase induction should be carefully evaluated for secondary consequences such as increased protease activity in relation to declining cell viability.

IV. CONCLUDING REMARKS

In recent years, there has been great advancement and achievement towards the production of therapeutic molecules, using plant cell cultures. This has been made possible because of better understanding of cell culture cultivating methods, and the accelerated pace of development and utilization of predictable and efficient processes that have emerged in recent times. As a result of these advances, yields of complex molecules such as paclitaxel have increased rapidly and led to scale-up to commercial production in 75 kl bioreactors. Without doubt, such successful industrial application of plant cell culture technology will trigger further research on the production of plant-derived active pharmaceutical compounds. Finally, the integration of newer technologies in plant cells, such as metabolic engineering, and better understanding of biochemical principles of scaling-up processes derived from suspension cell culture is anticipated to accelerate opportunities for commercializing the production of more useful therapeutic compounds.

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CHAPTER 4

Nanotechnology in the Detection and Control of Microorganisms

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I. INTRODUCTION

''Nano-'' denotes nanometer (10^{-9} meter). Richard Feynman introduced the concept of nanotechnology in 1959 in his prescient lecture ''There is Plenty of Room at the Bottom'' at a meeting of the American Physical Society (Khademhosseini and Langer, 2006). Since then, nanotechnology has developed into a multidisciplinary field of applied science and technology in the design, production, characterization, and application of nanoscale materials, structures, and devices (Fig. 4.1). In the nanoscale range, bulk materials exhibit different properties such as changes in conductivity and in surface-to-weight ratios (Kahn, 2006). Depending on their application, nanomaterials can be engineered into different shapes as particles (Elechiguerra *et al.*, 2005; Luo *et al.*, 2005a), rods (Al-Kaysi *et al.*, 2006; Wang *et al.*, 2005; Yi *et al.*, 2005), tubes (Elkin *et al.*, 2005; Lin *et al.*, 2007), wires (Stern *et al.*, 2006; Tilke *et al.*, 2003), films (Lu *et al.*, 2003), composites (Cioffi *et al.*, 2004, 2005) of varying sizes, ranging from a few to several hundred nanometers.

Nanomaterials can be produced from various bulk materials, with either organic or inorganic components as their major constituents. The nanomaterials to be discussed here will be divided into three categories: polymeric, semiconductor, and metallic (Liu, 2006). Organic polymeric nanomaterials include a large and diverse group of carbon nanotubes, emulsions, liposomes, dendrimers, and other organic polymers (Bianco *et al.*, 2005; Hofheinz *et al.*, 2005; Lee *et al.*, 2005; Park, 2002; Svenson and Tomalia, 2005; Tasis *et al.*, 2006). Metallic nanomaterials are composed

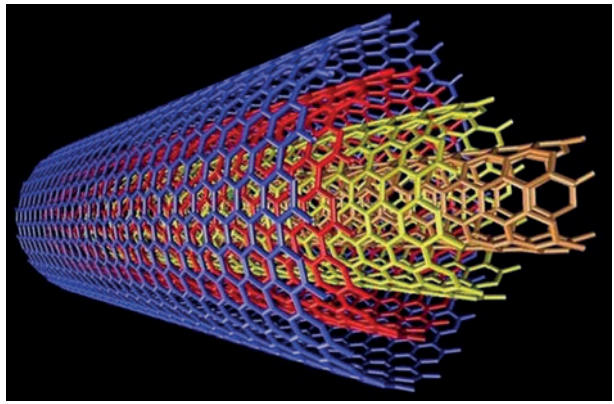

FIGURE 4.1 Image of a multiwalled nanotube constructed by Alain Rochefort (Ecole Polytechnique, Montreal, Canada) provided courtesy of Apparao Rao, Department of Physics and Astronomy, Clemson University.

of various elemental metals, metal oxides, and magnetic materials (Nolting *et al.*, 2003; Varshney *et al.*, 2005). Fluorescent quantum dots (QDs) are one of the most widely used semiconductor nanomaterials today (Alivisatos, 2004; Michalet *et al.*, 2005; So *et al.*, 2006).

Although nanotechnology is still new, nanomaterials have been widely used in electronics, magnetics and optoelectronics, energy and catalytic, biomedicine, pharmaceuticals, clothing, cosmetics, and environmental detection and monitoring (Liu, 2006; Penn *et al.*, 2003). Such applications are already affecting our daily lives, from drinking-water purification using nanofilters to stain-resistant ''nanopants'' made from fibers treated with fluorinated nanopolymers to food storage using the antibacterial and deodorizing effects of nanoparticles (Chou *et al.*, 2005; Jain and Pradeep, 2005; Savage and Diallo, 2005). In the biological and biomedical sciences and technology, nanomaterials have the potential to open new research avenues. However, most of the as-manufactured nanomaterials are hydrophobic and incompatible in biological environments. Over the past few years, however, solubilization and functionalization strategies have been developed to render nanomaterials water soluble (Lin *et al.*, 2004; Seydel, 2003).

Once rendered compatible with biological environments, nanomaterials can be conjugated with different biomolecules such as nucleic acids (Wu *et al.*, 2006; Zhang *et al.*, 2006), peptides and proteins (Elkin *et al.*, 2005; Winter *et al.*, 2001), antibodies (Elkin *et al.*, 2005; Jaiswal *et al.*, 2003), carbohydrates (Luo *et al.*, 2006; Rojo *et al.*, 2004), and antibiotics (Gu and Xu, 2006; Li *et al.*, 2005). Materials in the nanoscale range have a higher surface-to-volume ratio when compared with their microscale counterparts. This allows nanomaterials to be able to attach more copies of biological molecules, which confers greater efficiency. Bioconjugation not only further solubilizes nanomaterials, but also makes them suitable for various innovative biological applications, such as drug delivery (Balland *et al.*, 1996; Khademhosseini and Langer, 2006; Sahoo and Labhasetwar, 2003), cancer detection and diagnosis (McCarthy *et al.*, 2005; Shelley, 2006), labeling (Alivisatos *et al.*, 2005; Li and Ruckenstein, 2004), and biosensors (Chen *et al.*, 2004; Tapec *et al.*, 2002; Wang *et al.*, 2007). As one of the most important emerging research fields, nanoscience and nanotechnology also offer versatility in the control and detection of various microorganisms.

The increasing incidence of drug-resistant pathogens has caused great concern in the clinical environment (Neu, 1992; Walsh, 2000). Drug-resistant pathogens include methicillin-resistant *Staphylococcus aureus* (MRSA) (Mulligan *et al.*, 1993), vancomycin-resistant *Enterococci* (VRE) (Cetinkaya *et al.*, 2000), multiple drug-resistant *Mycobacterium tuberculosis* (MDR-TB), and penicillinase-producing *Neisseria gonorrhoeae* (PPNG) (Wright, 2003). Increasing drug-resistance has made the search for alternatives to

traditional means of pathogen control a high priority. Because of their excellent physiochemical properties, nanoscale materials have been investigated for antimicrobial activity so that they can be used as growth inhibitors, killing agents, or antibiotic carriers. Nanomaterials have a broad spectrum of biocidal and biostatic activity against a variety of microorganisms, including Gram-positive and Gram-negative bacteria (Gu *et al.*, 2003a, b,c; Luo *et al.*, 2005a; Morones *et al.*, 2005), bacterial endospores (Huang *et al.*, 2005a; Lin and Li, 2005; Stoimenov *et al.*, 2002; Wang *et al.*, unpublished), fungi (Melaiye *et al.*, 2005; Myc *et al.*, 2001), and viruses (Bender *et al.*, 1996; Elechiguerra *et al.*, 2005).

Rapid, sensitive, and accurate methods for detecting, separating, or identifying pathogens from air, water, food, biological, and medical samples are in constant demand to identify pathogenic infections and to combat bioterrorism threats (Tan *et al.*, 2004). For detection of small numbers of microorganisms in environmental or clinical samples, however, traditional methods need an amplification step based on growth in selective or differential media to increase cell numbers. These procedures are laborious and prolong the detection time, sometimes taking days to produce results that are often limited in their ability to distinguish between strains. Polymerase chain reaction (PCR) has been used in pathogen detection. However, it requires incubation and amplification to detect pathogens at low concentrations. In addition, the incubation and enrichment processes required for PCR may generate false positive results since it does not indicate viability. New detection approaches without time-consuming procedures offer obvious advantages in clinical diagnosis, environmental monitoring, food quality control, and homeland security. Various biofunctionalized nanomaterials have also been developed for rapid and sensitive detection, capture, and separation of pathogens (Edgar *et al.*, 2006; Lin and Sabri, 2005; Verma and Rotello, 2005; Willis, 2004; Zhao *et al.*, 2004a).

In this review, we discuss the current status of three major types of nanomaterials as they are applied in the control and detection of various microorganisms: (1) polymeric nanomaterials (nanoparticles, nanotubes, nanoemulsion) that are conjugated with different biofunctionalities (carbohydrates, antibodies, etc.), (2) fluorescent agents and the applications of semiconductor nanocrystals as fluorescent tag/label/probe for the rapid and sensitive identification and detection of pathogens in pure or mixed microbial cultures, (3) metallic nanomaterials such as Ag, TiO_2, and MgO that are used in antimicrobial applications. Because of the complex nature of nanoscience and nanotechnology involving multidisciplinary research fields (Chemistry, Material Sciences, Bioengineering, Physics, and Biological Sciences), we have limited our discussion to the antimicrobial strategies and mechanisms, rather than including the synthesis, analysis, and characterization of various nanomaterials.

II. POLYMERIC NANOMATERIALS

A. Carbohydrate-biofunctionalized polymeric nanomaterials

1. Carbohydrate-biofunctionalized polystyrene nanoparticles

Carbohydrates for antiadhesion therapy Carbohydrates serve as recognition molecules in the adhesion of microbes to host cells via carbohydrate–protein (Karlsson, 1999; Sharon and Lis, 1989, 1993) and carbohydrate–carbohydrate (Bovin, 1997; Hakomori, 1991) interactions. Carbohydrate analogues of host glycoconjugates have the potential to serve as antimicrobial adhesion agents in the prevention and treatment of infectious diseases. They could prevent microbes from attaching to, or could dislodge microbes from, host cell receptors. Carbohydrate-based antiadhesion therapy has been envisioned as a significant therapeutic potential as a substitute for, or augmentation of, conventional antibiotic treatment (Karlsson, 1998; Sharon and Ofek, 2000).

In the search for antiadhesion agents, various natural carbohydrates have been investigated including plant-derived constituents and human milk glycoconjugates (Burger *et al.*, 2000; Ruiz-Palacios *et al.*, 2003). Multiple copies of carbohydrate ligands displayed on the various carriers, including linear and branched polymers (Strong and Kiessling, 1999) and dendrimers (Turnbull *et al.*, 2002), have been developed to be more effective than their natural carbohydrate counterparts. Nanomaterials offer physicochemical properties not available in traditional carriers: high surface area and flexible nature (for nanotubes and nanowires). Thus, nanomaterials have been explored as a new class of carbohydrate carriers in antiadhesion therapy.

Carbohydrate-biofunctionalized polystyrene nanoparticles Attempts to develop antimicrobial adhesion agents using carbohydrate-biofunctionalized nanoparticles have included polystyrene nanospheres. Through a polyethylene glycol (PEG) tether, these polymeric nanoparticles (~150 nm in diameter) were covalently biofunctionalized with multiple mannose or galactose moieties (Qu *et al.*, 2005a,b). *Escherichia coli* strains of different carbohydrate specificities (mannose- or galactose-specific) were incubated with polymeric nanoparticles carrying two carbohydrate functionalities. Electron microscopy revealed that carbohydrate-biofunctionalized nanoparticles mediated the aggregation of bacterial cells (Fig. 4.2). The binding of carbohydrate nanoparticles to bacterial cells was via adhesin-receptor (carbohydrate–protein) interaction as demonstrated by the strong binding of mannosylated nanoparticles with a mannose-specific *E. coli* ORN178 strain and binding of galactosylated nanoparticles with a galactose-specific *E. coli* O157:H7 strain. There were minor cross reactions, which could be explained by the fact that a bacterium could express more than one type of adhesin (Sharon and Ofek, 2000). Clumps of thousands of cells were

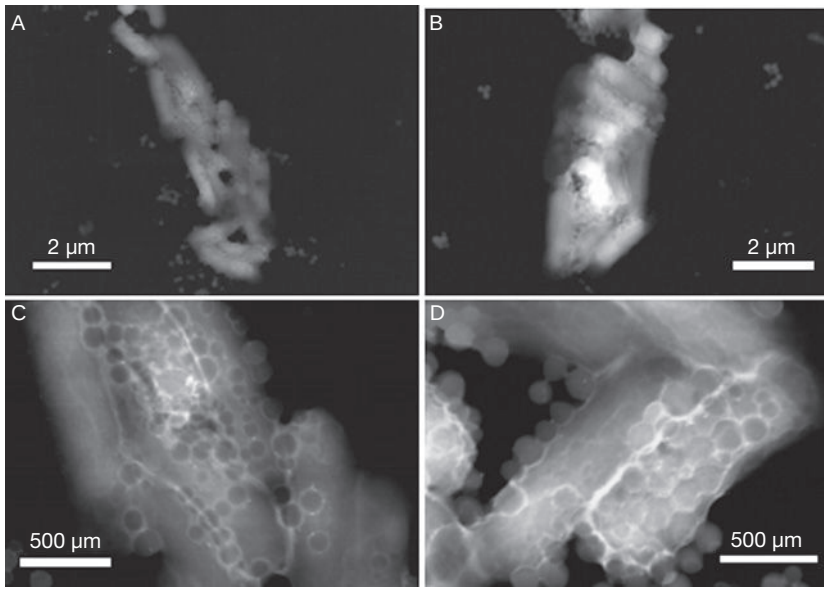

FIGURE 4.2 Low (A, B) and high (C, D) magnification transmission electron micrographs of the agglutination of *E. coli* ORN178 by mannosylated nanoparticles. (Reproduced from Qu *et al.*, 2005a with permission. Copyright © 2005 American Scientific Publishers.)

apparent under fluorescent microscopy. Measurements of colony forming units (CFU) showed up to 99% of CFU reduction under a putative optimal nanoparticles-to-bacteria ratio of 33.3 μg/10^6 cells (Luo *et al.*, 2005a).

Mannosylated polymeric nanoparticles inhibited the binding of *Campylobacter jejuni* to a colon cancer cell line Caco-2 (Luo, 2006). These carbohydrate nanoparticles also reduced *C. jejuni* populations about 70% in turkey poults when the birds were gavaged with mannose nanoparticles (Franklin *et al.*, 2003). No apparent cytotoxicity was observed when these polymeric nanoparticles were tested *in vitro* and *in vivo* (Franklin *et al.*, 2003; Molugu *et al.*, 2006), indicating the clinical potential of these carbohydrate-biofunctionalized nanoparticles for antimicrobial adhesion treatment.

2. Carbohydrate-biofunctionalized nanomaterials for sugar microarrays

Carbohydrate microarrays were first reported in 2002 (Houseman and Mrksich, 2002; Wang *et al.*, 2002) following the successful development of DNA (DeRisi *et al.*, 1997; Marshall and Hodgson, 1998) and protein-coated chips (MacBeath and Schreiber, 2000; Zhu and Snyder, 2001). Earlier studies of model systems have found that carbohydrate arrays are useful

for molecule detection in the recognition of carbohydrate–protein interactions (Schwarz *et al.*, 2003; Wang, 2003). As an attempt to explore the capability of carbohydrate nanomaterials in cellular detection for microbe recognition, carbohydrate microarray chips were fabricated by spotting polymer (Luo *et al.*, 2005b) and polymeric nanoparticle probes (Luo *et al.*, unpublished) onto nitrocellulose membrane FAST? slides. *E. coli* ORN178 bound specifically to mannose on microarray chips, while strains *E. coli* ORN208 and O157:H7 did not. This binding was mannose-concentration dependent and inhibited by pre-exposing *E. coli* ORN178 to a high concentration of soluble mannose. Because of their high throughput nature, it was envisaged that carbohydrate microarrays could be used in clinical applications to identify specific pathogens. However, the development of carbohydrate microarrays is currently impeded by the availability of some adhesin-specific carbohydrates. Carbohydrate-biofunctionalized nanomaterials may be one of the most economical ways to suit this need.

B. Carbohydrate- or antibody-conjugated nanotubes

1. Carbohydrate-biofunctionalized carbon nanotubes

Carbon nanotubes represent a unique class of nanostructures. Recently, nanotubes biofunctionalized with multiple copies of biomolecules (e.g., carbohydrate, antibody) were prepared for antimicrobial applications and detections. Galactose biofunctionalized single wall nanotubes (Gal-SWNTs) act as highly efficient aggregating agents via their multivalent interaction with galactose-binding surface proteins on pathogenic *E. coli* O157:H7 cells (Gu *et al.*, 2005). Multiple-walled carbon nanotubes biofunctionalized with multiple copies of divalent dendrimeric galactose cluster (Gal_2-MWNTs) were much more soluble when compared with their monovalent counterparts Gal-MWNTs, probably because of the availability of more branched functional carbohydrates (Gu and Lin, 2006). The interactions between *E. coli* O157:H7 and MWNTs biofunctionalized with these two sugar configurations were evaluated qualitatively and quantitatively. Gal_2-MWNTs had higher antiadhesion capability than Gal-MWNTs, as indicated by larger bacteria-nanotube aggregates and higher CFU reduction (Luo *et al.*, 2006).

Carbohydrate-biofunctionalized nanotubes have also captured and detected bacteria. A small amount (10 μl) of the galactose biofunctionalized MWNTs described earlier could detect $\sim 10^4$ bacterial cells (Luo *et al.*, 2006), a sensitivity which is comparable to some of the existing bacterial detection techniques using fluorescent polymer (Disney *et al.*, 2004) and immunological assays (Yu *et al.*, 2002).

Carbohydrate-bearing nanotubes were very effective in agglutinating Gram-positive bacterial endospores as well as Gram-negative vegetative

cells. In a unique sugar-nanotube system for the aggregation of *Bacillus anthracis* endospores (Wang *et al.*, 2006), the addition of Ca^{2+} enabled carbohydrate nanotubes (Man- or Gal-SWNTs) to form large spore aggregates, each composed of hundreds of spores captured by a ''spider web'' of sugar nanotubes (Fig. 4.3). The interaction between anthrax spores and sugar nanotubes was speculated to be carbohydrate–carbohydrate, mediated by Ca^{2+}, between the carbohydrates on the anthrax spore surface and the nanotube–sugar complexes. This property has potential in reducing inhalation risk during bioterrorism and biowarfare.

2. Antibody-conjugated Carbon nanotubes

Carbon nanotubes have been conjugated with antibody to form immunonanotubes for microbial detection and control. Bovine serum albumin (BSA)-SWNT conjugates were prepared by a carbodiimide-activated amidation reaction of the nanotube-bound carboxylic acids with pendant amino moieties on BSA. Goat anti-*E. coli* O157 immunoglobulins were directly adsorbed on the SWNT-BSA to form immuno-SWNTs. These immuno-SWNTs recognized pathogenic *E. coli* O157:H7 cells through specific antibody–antigen interactions (Elkin *et al.*, 2005). The application of Carbon nanotubes for immunomagnetic separation of pathogenic bacteria will be discussed in Section IV.C.1.

C. Chitosan nanoparticles

Chitosan is a natural, nontoxic polysaccharide derived by the deacetylation of chitin. Because of their antimicrobial activity, chitosan and its derivatives have attracted substantial interest. Qi *et al.* (2004) prepared

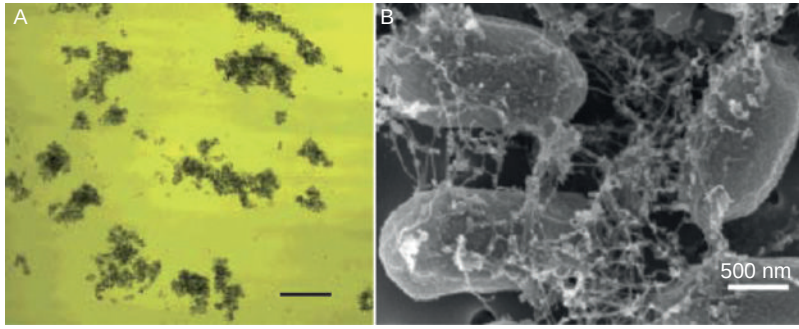

FIGURE 4.3 Optical (A) and electron (B) micrographs of the aggregation of *B. anthracis* spores induced by the Ca^{2+}-mediated binding with mannose biofunctionalized SWNTs. (Reproduced from Wang *et al.*, 2006 with permission. Copyright © 2006 American Chemical Society.)

nanoparticles via the ionic gelation of chitosan. Chitosan nanoparticles and copper-loaded nanoparticles were evaluated *in vitro* against *E. coli*, *Salmonella choleraesuis*, *S. typhimurium*, and *S. aureus* using minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) criteria. The chitosan nanoparticles and copper-laden nanoparticles had significantly higher antibacterial activity than that of chitosan alone. The enhanced antibacterial activity was attributed to their small size and positive charge. Exposure of bacteria to chitosan nanoparticles led to disruption of cell membrane and the leakage of cytoplasm. Copper nanomaterials for microbial control are further discussed in Section IV.A.3.

The antibacterial properties of bone cement impregnated with chitosan nanoparticles were tested against *S. aureus* and *Staphylococcus epidermidis* (Shi *et al.*, 2006). A thousand-fold reduction in the number of viable *S. aureus* was achievable with chitosan nanoparticles. With the addition of chitosan alone to the cement, it was found that the number of viable *S. aureus* cells decreased only by 50%. The chitosan derivatives were equally effective against *S. epidermidis* and exhibited higher antibacterial activity than the chitosan powder alone. Chitosan nanoparticles showed no cytotoxic effect in mouse fibroblast MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide cell proliferation) assay. Chitosan nanoparticles also show promise as vaccine vehicles. For example, BSA-loaded chitosan nanoparticles encapsulated in vesicles have been useful for oral immunization (Jain *et al.*, 2006), which is discussed in next section.

D. Nanomaterials for vaccine developments

Nanomaterials have been tested as adjuvant (Kim *et al.*, 1999), carrier (Baudner *et al.*, 2002), and even vaccine (Raman *et al.*, 2005). Most pathogens invade their hosts at the mucosal surfaces, the first antimicrobial barrier. Mucosal vaccinations offer the advantage of blocking the pathogens at the portal of entry. *Helicobacter pylori* lysate-loaded poly (D, L-lactide-coglycolide) (PLG) nanoparticles (mean diameter of less than 1 μm). Oral immunizations of mice with *H. pylori*-PLG nanoparticles induced the antibody isotype/subclasses similar to those obtained with cholera toxin (CT) adjuvants and resulted in Th2-type responses, apparently because the Th2 cell-secreted cytokines supported IgG1 and IgA synthesis (Kim *et al.*, 1999).

E. coli heat-labile enterotoxin (LT) LTK63 mutant was used as a mucosal adjuvant in ''supramolecular biovectors'' (nanoparticles of cationic cross-linked polysaccharides surrounded by a lipid bilayer, SMBV) (Baudner *et al.*, 2002). Delivery of conjugated group C meningococcal vaccines in the presence of the LTK63 mutant and SMBV nanoparticle significantly enhanced the immunogenicity and protective efficacy of the intranasal

vaccine. Highest antibody titers were obtained when both the LTK63 and the SMBV were administered with the conjugated vaccine.

The *in vitro* and *in vivo* immune stimulation by BSA-chitosan nanoparticle-encapsulated vesicles following oral immunization resulted in significantly higher serum IgG titers and secretory IgA (sIgA) levels in mucosal secretions (Jain *et al.*, 2006). Nanoparticles encapsulated in vesicles (liposomes and niosomes) confer enhanced acid resistance.

When epitopes alone are used as vaccine, they have weak immunogenic signatures, which render them less effective. Alternatively, attenuated vaccines are shadowed by potential revertants that might cause the onset of the diseases that they seek to prevent. Inactivated or recombinant vaccines may cause allergic reactions in hosts. Inert nanomaterials could serve as an ideal epitope carrier/presenter in vaccine applications because of their organized structures that display epitopes in multivalent and clustered formations for enhanced immunogenicity.

E. Other polymeric nanomaterials

Nanoemulsions (micellar lipid nanoparticles in a uniform population of droplets ranging in diameter from 400 to 800 nm) were tested for antimicrobial activities against bacteria, viruses, and fungi (Hamouda *et al.*, 2000; Myc *et al.*, 2001). A nanoemulsion, $X8W_{60}PC$, exhibits strong fungicidal effects on *Candida albicans* and *Candida tropicalis*. Because of coalescence with nanoemulsion droplets that surrounded the cell, yeast cells treated with nanoemulsion lose their round shape. Overall, all tested fungi were susceptible to the nanoemulsions at a concentration below 0.1%. Nanoemulsions are nontoxic to the skin, mucous membranes, and gastrointestinal tissue of tested animals at fungicidal concentrations (Myc *et al.*, 2001).

Polymeric nanoparticles have also attracted interest as drug vehicles capable of enhancing intracellular drug delivery (Fattal *et al.*, 1989). For example, polyisohexylcyanoacrylate (PIHCA) nanoparticles (187 ± 13 nm) were bound to ampicillin for use in the treatment of experimental *Listeria monocytogenes* infection in congenitally athymic nude mice (Youssef *et al.*, 1988).

Monocyte/macrophage (Mo/Mac) in the mononuclear phagocyte system plays important roles in the pathogenesis of human immunodeficiency virus (e.g., as a reservoir for the virus). To effectively combat infected Mo/Mac cells, nanoparticles were prepared by emulsion polymerization from polyhexylcyanoacrylate (PHCA) and loaded with zalcitabine or saquinavir (a nucleoside analogue and a HIV protease inhibitor, respectively). Nanoparticles with zalcitabine were ineffective, but saquinavir-loaded nanoparticles caused a tenfold increase in activity compared with that of the free drug. Even when the infected Mo/Mac

had reached their highest level of virus production, a 35% reduction of HIV antigen production was obtained with the nanoparticle-bound drug (Bender *et al.*, 1996).

III. FLUORESCENCE DETECTION OF MICROORGANISMS

Microscopy is probably the single most used research technique in microbiology. However, electron microscopy cannot be used on living organisms and optical microscopes cannot resolve individual molecules. Fluorescence labeling has been used to track molecules of interest in various biological processes. Conventional organic dyes and fluorescent proteins have several disadvantages: fast photobleaching, low signal-to-noise ratio, and broad emission spectra. Nanotechnology offers means to overcome these shortcomings by engineering nanoparticles filled with thousands of organic fluorophores (Tapec *et al.*, 2002; Zhao *et al.*, 2004b). Although dye-doped methods still rely on the properties of conventional organic dyes, improved inorganic fluorophores based on the photophysics of colloidal semiconductor nanocrystals have been generated (Bruchez *et al.*, 1998; Chan and Nie, 1998). Several research groups have successfully linked fluorescent nanoparticles to peptides, proteins, and nucleotides for microbial detection. In this section, we review the current status of fluorescent labeling in the detection and control of various microorganisms using nanotechnology.

A. Dye-doped silica nanoparticles

Dye-doped silica nanoparticles have proven useful in many applications (Bagwe *et al.*, 2003; Tan *et al.*, 2004). Dye-doped silica nanoparticles conjugated with antibody provided a rapid bioassay for single bacterial cell detection (Zhao *et al.*, 2004a). Fluorescent silica nanoparticles (60 nm diameter) were synthesized by encapsulating thousands of RuBpy dye molecules inside a silica matrix. The surface of these fluorescent nanoparticles was conjugated with monoclonal antibodies specific for *E. coli* O157:H7. Compared with conventional fluorescent-based immunological detection techniques, which usually use only one or a few dye molecules attached to a single antibody, fluorescent-bioconjugated silica nanoparticles achieved an amplification effect and increased the detection sensitivity to a single bacterium. Thousands of these fluorescent nanoparticles attach to each bacterium. Targeted bacterial cells thus emitted stronger fluorescent signals and could be easily detected using a normal spectroflurometer in a conventional plate-based immunological assay, or could be accurately enumerated using a laboratory-made flow cytometer within a minute of sample preparation. The detection capability within 20 min

was confirmed by plate-counting and optical methods. Furthermore, by linking different antibodies to the nanoparticles, mixtures of bacteria and spores could be detected simultaneously.

B. Quantum dots (QDs) for fluorescent detection

1. Fluorescent QDs

QDs, also known as colloidal semiconductor nanocrystals, are novel inorganic fluorescence labeling agents (Alivisatos *et al.*, 2005; Gao *et al.*, 2005). They are generally composed of materials in the periodic groups of II–VI (e.g., CdSe) or III–V (e.g., InP) and are typically less than 10 nm in diameter. A bare QD is commonly passivated by an inorganic layer to compose a core-shell structure (e.g., CdSe/ZnS). While the core defines the fluorescence optical and electronic properties of the particles, the outside shell layer increases the quantum yield and protects the core from degradation in a physiologically aggressive environment to enhance its photostability. The core-shell QD can be coated with an outer layer of amphiphilic polymer and further conjugated with biofunctional groups (Fig. 4.4). Bioconjugation not only further passivates the core-shell QD, but also renders it hydrophilic, making it compatible in biological environments.

QDs behave as if they were individual atoms: they can absorb light energy, which excites their internal electrons up to higher energy levels, then release it as emitted light. Compared with conventional organic fluorophores, QDs exhibit advantageous photophysical properties, including strong brightness, resistance to photobleaching, larger Stokes shift value, composition- and size-dependent absorption and emission,

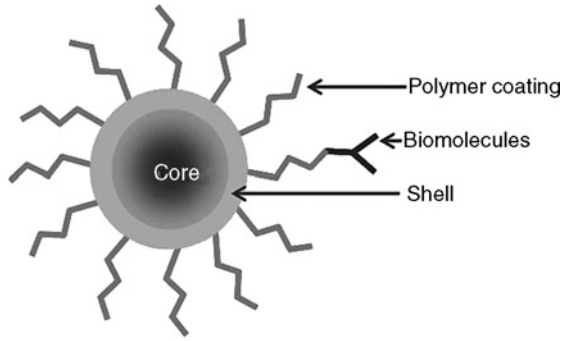

FIGURE 4.4 A typical structure of the quantum dot core-shell structure (courtesy of Dr. Li Cao, Department of Chemistry of Clemson University). The inner core-shell may be further coated with a layer of amphiphilic polymers to render the nanoparticle hydrophilic. Biomolecules (e.g., antibody as shown in the figure) solubilize and biofunctionalize the nanoparticle.

and multiple targeting capabilities (Bailey and Nie, 2003; Jaiswal *et al.*, 2003; Mattoussi *et al.*, 2000; Wu *et al.*, 2003). These properties of QDs enable them to be used in biological systems over extended time periods for single particle detection. For a more detailed description of QD physical chemistry and characterization, see reviews by Alivisatos *et al.* (2005), Michalet *et al.* (2005), and Smith *et al.* (2004)). Here, we will limit discussion to the QD applications for various microorganisms.

Fluorescent imaging of various eukaryotic cells with QDs has been well established (Gao *et al.*, 2004; Wu *et al.*, 2003). Many recent reports have described QDs linked to biorecognition molecules such as streptavidin (Wu *et al.*, 2003), peptides (Winter *et al.*, 2001), antibodies (Jaiswal *et al.*, 2003; Tada *et al.*, 2007), or small-molecule ligands (Derfus *et al.*, 2004a; Lidke *et al.*, 2004) for fluorescent labeling of fixed (Bruchez *et al.*, 1998; Pathak, 2001) or live cells (Hoshino *et al.*, 2004; Michalet *et al.*, 2005). However, the documented use of semiconductor QDs in detecting or imaging microorganisms has lagged behind that of eukaryotic cells. Jaiswal *et al.* (2003) were the first to report that amoeba, *Dictyostelium discoideum* (AX2), could be fluorescently labeled using QDs. Later reports have included fungi (Kloepfer *et al.*, 2003), protozoa (Ferrari and Bergquist, 2007; Lee *et al.*, 2004; Zhu *et al.*, 2004), viruses (Edgar *et al.*, 2006), bacteria (Dwarakanath *et al.*, 2004; Hahn *et al.*, 2005; Hirschey *et al.*, 2006; Kloepfer *et al.*, 2003, 2005; Otsuka *et al.*, 2004; Su and Li, 2004; Tully *et al.*, 2006; Wang *et al.*, 2007; Yang and Li, 2005, 2006), and complex microbiological environments, such as biofilms (Chalmers *et al.*, 2007).

2. QDs for external and internal labeling of microbes

Quantum dots as strain- and metabolism-specific microbiological labels

Kloepfer *et al.* (2003) first reported the use of biologically conjugated CdSe QDs (3–10 nm) to fluorescently label various bacteria and fungi. While unconjugated QDs were shown to adhere to some bacterial strains, external fluorescent labeling specific to Gram-positive bacteria was achieved using WGA (wheat germ agglutinin lectin)-conjugated QDs. Labeling was detectable visually, microscopically, and spectroscopically after incubation of WGA-conjugated QDs with live or dead bacteria. QD conjugated to human transferrin was used for metabolism-specific internal labeling for various fungi (e.g., *Schizosaccharomyces pombe, Penicillium chrysogenum*) and bacteria (e.g., *S. aureus*) that are able to use transferrin-bound iron. Bacterial cell surface molecules, such as glycoproteins, make excellent targets for WGA-conjugated QDs for external labeling. The internal labeling takes advantage of the ability of pathogenic bacteria to harvest iron from human transferrin. There were many unexplained spectral changes upon QD binding to the microorganisms, including broadening of absorption peaks, blue-shifting of fluorescence peaks, and increasing emission intensities. Fluorescence emission blue shifts were also

observed in another study when CdSe/ZnS QDs conjugated to antibodies or DNA aptamers that were bound to bacteria (Dwarakanath *et al.*, 2004).

Kloepfer *et al.* (2005) synthesized adenine- and AMP-conjugated QDs. Internal labeling of *Bacillus subtilis* and *E. coli* was dependent upon purine-processing mechanisms, as auxotrophic mutants lacking adenine deaminase or adenosine phosphoribosyltransferase demonstrated a different fluorescent signal than did wild-type strains. The entry of adenine- and AMP-conjugated QDs into bacteria was size dependent, only the particles <5 nm in diameter were able to internally label bacteria.

Other internal quantum dot labeling techniques While both studies by Kloepfer *et al.* (2003, 2005) for internal microbial labeling described earlier are metabolism-specific, Li *et al.* (2004) achieved internal QD labeling using chemical treatment of bacterial cells. In their study using QD probes to explore the mechanism of competence development in *E. coli* HB101, QDs of 3–4 nm were taken up after bacterial membrane disruption by Ca^{2+}. Hirschey *et al.* (2006) synthesized a series of CdSe/CdS QDs functionalized with citrate, isocitrate, succinate, and malate. Hydrodynamic diameters of these organic acid-stabilized QDs were 3.8, 4.0, 4.5, and 6.0 nm, respectively. When incubated with *E. coli* (strains K12 and XL10), these QDs could enter bacterial cells, as established by epifluorescence and confocal fluorescence scanning microscopy, fluorimetry, and UV–vis spectroscopy on whole and lysed bacterial cells. These QDs were also used as co-stains along with two organic fluorescent dyes, either DAPI (4′, 6-diamidino-2-phenylindole dihydrochloride), which specifically stains the nuclear material within a cell, or FM4–64, which stains membranes. In both cases, QDs appeared inside the cells.

3. QDs in immunofluorescent detection

Antibody-conjugated QDs exhibit enhanced antibacterial effects versus unconjugated QDs (Dwarakanath *et al.*, 2007). However, more studies have incorporated antibody and QDs for immunofluorescent detection of various microbes. In immunofluorescent detection systems, antibody normally provides the binding specificity to targeted microbes while QDs that labeled the targeted microbes provide the fluorescence for detection. A QD-based secondary immunofluorescence assay was developed to detect a *Mycobacterium bovis* Bacillus Calmette–Guerin (BCG) strain in a species-specific manner (Otsuka *et al.*, 2004). Samples were mounted on a glass cover slip, air dried, and fixed with 2% glutaraldehyde. Antiserum from rabbits immunized with heat-killed BCG was applied, followed by a QD (655 nm) goat $F(ab')_2$ anti-rabbit IgG conjugate. Under confocal laser scanning microscopy, BCG strains were found to be labeled in red, whereas *Mycobacterium smegmatis* remained unlabeled.

Quantum dot fluorescent labeling via biotin–streptavidin conjugation Otsuka *et al.* (2004) took advantage of specific binding between primary and secondary antibodies, while other studies made use of the specific binding between biotin and streptavidin for QD-based secondary immunofluorescence assays. Zhu *et al.* (2004) detected *Cryptosporidium parvum* and *Giardia lamblia* protozoan cells using QD–streptavidin conjugates. Two strategies effectively labeled these protozoan cells: (1) the target cells were first bound with biotinylated antibodies before conjugation of streptavidin-coated QDs to the cell-attached antibodies; (2) streptavidin-coated QDs were first linked with biotinylated antibodies before the addition to the target cells. QD labeling exhibited better photostability and higher brightness than commercial staining kits. Both strategies achieved a similar maximal signal-to-noise ratio. The first strategy described earlier was successfully used in a separate study to detect *C. parvum* oocysts, its transmissive stage (Lee *et al.*, 2004). Later, the principle of streptavidin-coated QDs to bind with biotinylated antibodies, specific for targeted microbes, was also extended to detect *E. coli* O157:H7 (Hahn *et al.*, 2005) and *L. monocytogenes* (Tully *et al.*, 2006).

QDs in immunomagnetic separation Immunomagnetic separations coupled with QD fluorescent labeling has been employed for the control of various microbes. *E. coli* O157:H7 cells were captured by magnetic beads coated with anti-*E. coli* antibody, and biotin-conjugated anti-*E. coli* antibodies were added to form sandwich immunocomplexes. After magnetic separation, the immunocomplexes were labeled with streptavidin-conjugated QDs. When compared with FITC-based labeling, labeling with QDs was at least 100 times more sensitive (Su and Li, 2004). Immunomagnetic separation coupled with QD florescent labeling was used for the detection of *S. typhimurium* (Yang and Li, 2005), the simultaneous detection of *E. coli* and *S. typhimurium* (Yang and Li, 2006), and the detection of *L. monocytogenes* (Wang *et al.*, 2007).

4. QDs as luminescent probes to achieve single-cell resolution of oral biofilms

Most of the studies using QDs as fluorescent probes for microbial detection were secondary immunofluorescent assays as already described. While indirect methods are more sensitive, primary detections can simultaneously recognize multiple unique targets and thus are more suitable to study complex situations such as interspecies interactions and species diversity. Formation of oral biofilms (multispecies of oral bacterial communities growing at interfaces) is a natural mode of growth and interaction for numerous bacteria. In their nascent stages of development, better insight into the spatial relationship between different species and how species diversity increases over time can elucidate

the role of interspecies interactions. Chalmers *et al.* (2007) conjugated few polyclonal and monoclonal antibodies to the QD surface and developed QD-based primary immunofluorescence to study oral biofilms. They effectively detected distinct target bacteria with single-cell resolution in planktonic cultures of oral bacteria and biofilms under *in vitro* and *in vivo* conditions. The QD-based technique and the conventional dye-based primary immunofluorescence approach were complementary tools in immunofluorescence applications. QD-based immunofluorescence should help to define bacterial community architecture and to facilitate investigations of interactions between bacterial species in these communities.

5. QDs in bacteriophage assays for bacterial detection

Edgar *et al.* (2006) described a sensitive and rapid method for bacterial detection using biotin-tagged bacteriophage and streptavidin-linked QD nanocomplexes. *E. coli*-specific coliphage T7 was genetically engineered to express a viral surface protein (gp10A). The ''nonbiotinylated reagent coliphage'' then infected and reproduced in sensitive wild-type host *E. coli*. Progeny viruses were biotinylated (biotin gets attached to the genetically expressed gp10A protein on the phage surface) by *E. coli*'s biotin-ligase protein (BLP) BirA. The biotinylated coliphages were able to selectively bind streptavidin-coated QDs. The resulting phage–QD complexes could be detected and counted using spectroscopy, flow cytometry, or microscopy (Fig. 4.5). Because each infected bacterium can result in a release of 10–1000 phages that can be readily detected by the use of QDs, this phage-based assay greatly increases detection sensitivity and provided specific detection of as few as 10 bacterial cells/ml within an hour.

Although employed only in a model system (coliphage T7-*E. coli*), phage-based techniques are believed to be applicable to any bacteria susceptible to specific phages because biotinylation is a highly conserved pathway in nature (biotin is present in all living cells). Moreover, this method can be expanded to simultaneously detect a variety of bacterial strains in a single biological sample by conjugating different bacteriophages with QDs having different emission spectra. Phage-based QD fluorescence assay was predicted to be useful especially for the detection of bacterial strains that are slowly growing or are highly infectious. Furthermore, while traditional pathogen detection techniques such as immunoassays and nucleic acid-based amplification technologies (e.g., PCR) do not distinguish between live and dead bacteria unless a lengthy enrichment step is included, phage-based detection assay has the advantage of identifying living microorganisms, which is of great concern in food and pharmaceutical industries.

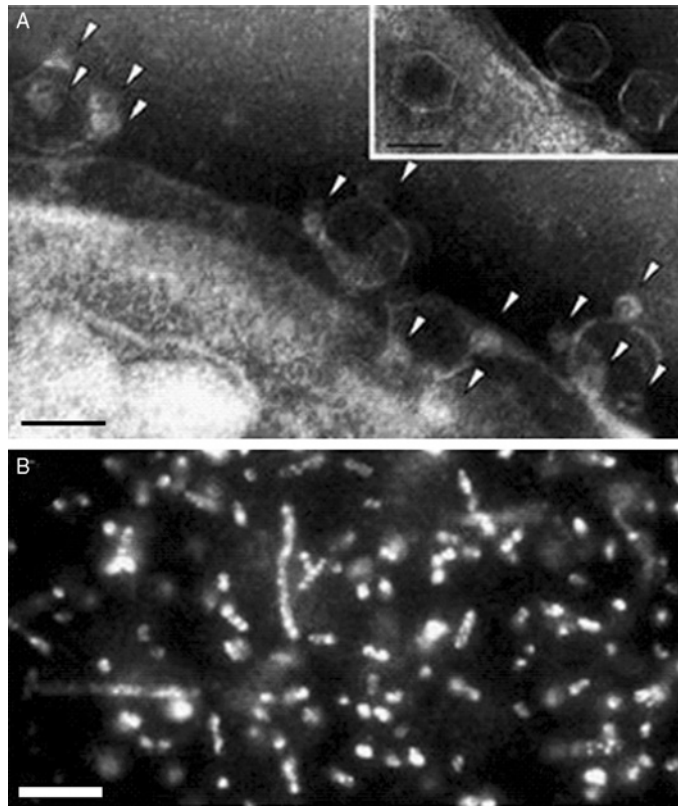

FIGURE 4.5 (A) TEM image of T7-biophage bound to streptavidin-functionalized QDs (arrowheads). Control T7-myc phage were not biotinylated and lack conjugated QDs (inset). Scale bar = 50 nm. (B) Fluorescent micrograph of phage–QD complexes bound to *E. coli* cells with 100-fold excess of biotinylated phage. Scale bar = 2 μm. (Reproduced from Edgar *et al.*, 2006 with permission. Copyright © 2006 National Academy of Sciences, U.S.A.).

C. Carbon-based fluorescent nanoparticles

Although useful in various biological applications, CdSe-based QDs have raised concerns about their potential cytotoxicity (Derfus *et al.*, 2004b; Hardman, 2006; Kirchner *et al.*, 2005). Carbon-based fluorescent nanoparticles (called as ''carbon dots'' or ''C-dots'') retain many physicochemical properties of CdSe-based QDs such as photostability and spectral characteristics and are safer from the biological standpoint. Although C-dots are still in their early stage of development, they have already been used to label eukaryotic (Sun *et al.*, 2006; Cao *et al.*, in review) and bacterial cells (Sun *et al.*, 2006).

IV. METALLIC NANOMATERIALS

Metallic nanomaterials are a unique class of materials for the development of novel devices in biological, biomedical, and pharmaceutical applications. Among the most widely studied are elemental metals (i.e., gold, silver, copper, etc.), metal oxides (i.e., MgO, ZnO, TiO_2, etc.), and magnetic compounds. These inorganic nanomaterials can be used to control a variety of microorganisms, including fungi (Cioffi *et al.*, 2004, 2005; Kim *et al.*, 2007), Gram-negative and Gram-positive bacteria (Esteban-Cubillo *et al.*, 2006; Son *et al.*, 2004), bacterial endospores (Stoimenov *et al.*, 2002), and viruses (Elechiguerra *et al.*, 2005). Some of the inorganic nanomaterials have inherent microbicidal or microbistatic effects. Others could be used as carriers for antibiotics and antibodies or for drug delivery or pathogen detection or both. Antimicrobial effects of various nanomaterials are dependent on particle size (Lok *et al.*, 2007; Panacek *et al.*, 2006), shape (Morones *et al.*, 2005; Pal *et al.*, 2007), and dose/target ratios (Shrivastava *et al.*, 2007). Putative antimicrobial mechanisms include the alteration of microbial membrane morphology to increase permeability (Sondi and Salopek-Sondi, 2004), generation of oxidizing power (Kim *et al.*, 2007; Lok *et al.*, 2007), and modulation of bacterial signal transduction, which inhibits cell growth (Shrivastava *et al.*, 2007).

These metallic nanomaterials have advantages over conventional chemical antimicrobial agents in that microbes are not likely to develop resistance as they do against conventional and narrowly targeted antibiotics. The metal attacks a broad range of targets, which means that microbes would have to simultaneously develop multiple mutations to protect themselves (Pal *et al.*, 2007).

A. Elemental metal nanomaterials

1. Silver nanoparticles

Silver has been known for its biological effects since antiquity (Silver and Phung, 1996). Although silver exhibits strong toxic effects against prokaryotes, it has low toxicity against human cells. Silver-based compounds containing ionic silver (Ag^+) (Kim and Kim, 2006), metallic silver (Ag^0), and more recently silver nanoparticles have been tested against a variety of microbes (Aymonier *et al.*, 2002; Jiang *et al.*, 2004; Kim *et al.*, 2007; Lok *et al.*, 2006). Sondi and Salopek-Sondi (2004) studied the activity of silver nanoparticles against *E. coli*. In that study, Electron microscopy revealed ''pits'' in the cell walls, which is caused by the accumulation of silver nanoparticles, resulting in alteration of membrane morphology and increase in permeability, eventually leading to cell death (Fig. 4.6).

Silver nanoparticles interact with *E. coli*, *Vibrio cholera*, *Pseudomonas aeruginosa*, and *S. typhi* in a particle size-dependent fashion (Morones *et al.*, 2005).

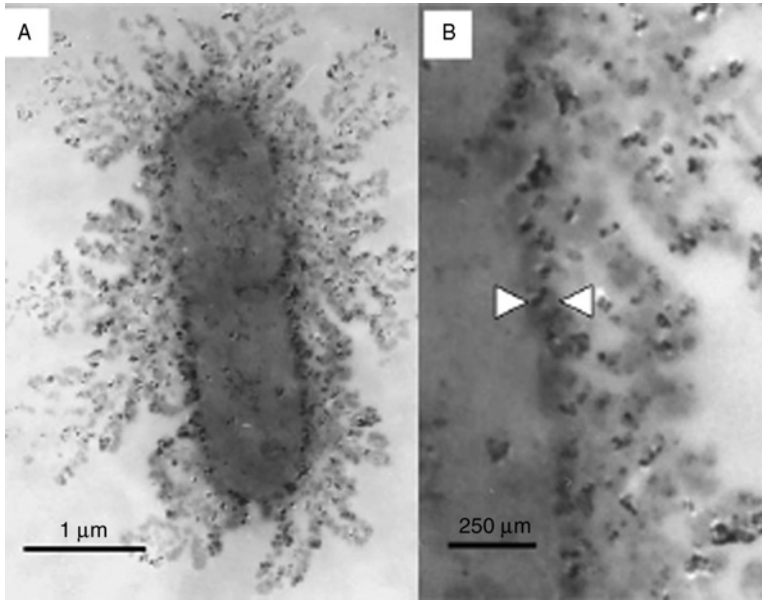

FIGURE 4.6 TEM images of *E. coli* treated with silver nanoparticles (A) and enlarged view of the incorporation of silver nanoparticle into the cell membrane structure (B). (Reproduced from Sondi and Salopek-Sondi, 2004 with permission. Copyright © 2004 Elsevier Inc.).

Nanoparticles in the range of 1–10 nm most effectively bind to the surface of the cell membrane. Although *P. aeruginosa* and *V. cholera* exhibited more resistance than *E. coli* and *S. typhi* at low silver nanoparticle concentrations, growth of all bacteria was inhibited above 75 µg/ml of nanoparticle concentration. Scanning tunneling electron microscopy (STEM) and X-ray energy dispersive spectrometry (EDS) revealed silver nanoparticles both at the surface of cell membranes and inside the bacteria. Bactericidal effects were attributed to the disturbance of membrane permeability, to respiration, and to interaction with DNA.

Silver nanoparticles can be generated using a one-step modified Tollens procedure (reduction of $(Ag(NH_3)_2)^+$). Using various reducing saccharides, silver colloid particles ranging form 25 to 380 nm were synthesized (Kvitek *et al.*, 2005; Panacek *et al.*, 2006). Silver particles of 44, 50, 25, and 35 nm were obtained using two monosaccharides (glucose and galactose) and two disaccharides (maltose and lactose), respectively. The nanoparticles were tested against *S. aureus* strains, *S. epidermidis* strains, *Enterococcus faecalis*, and *Klebsiella pneumoniae*. Particles synthesized with disaccharides had higher antibacterial activity than those synthesized with monosaccharides in a size-dependent fashion (Panacek *et al.*, 2006). Pal *et al.* (2007) were the

first to investigate shape-dependent interaction of silver nanoparticles with *E. coli*. Truncated triangular silver nanoplates displayed the strongest biocidal action, compared with spherical or rod-shaped nanoparticles or $AgNO_3$.

Silver nanoparticles also bind to HIV-1; those in the range of 1–10 nm are found to be most effective. The silver nanoparticles blocks the gp120 subunit of the viral envelope glycoprotein, and prevents HIV binding to host cells (Elechiguerra *et al.*, 2005).

Silver nanoparticles can also be loaded onto foam (Jain and Pradeep, 2005), fibers (Son *et al.*, 2004, 2006), polymer films (Ho and Tobis, 2004), and even textile fabrics (Lee *et al.*, 2003). Silver nanoparticles can serve as a vehicle for antibiotic delivery (Li *et al.*, 2005), as disinfecting filters (Jain and Pradeep, 2005) and as coating materials (Li *et al.*, 2006). However, silver nanoparticles incorporated into implantable materials to reduce nosocomial infection by *S. epidermidis* yielded mixed results (Furno *et al.*, 2004).

2. Gold nanoparticles

Gold nanoparticles are routinely used as drug carriers in antimicrobial drug delivery systems. Tom *et al.* (2004) used ciprofloxacin (cfh) to coat gold nanoparticles; the release of cfh from nanoparticles is dependent on the size of nanoparticles, with faster desorption from smaller particles. Vancomycin-capped gold nanoparticles (Au@Van) had enhanced antibacterial activities *in vitro* against VRE strains and *E. coli.* The increased activities against VRE were attributed to multivalency of vancomycin and the binding of nanoparticles to the outer membrane of *E. coli* (Gu *et al.*, 2003a). Grace and Pandian (2007) developed gold nanoparticles coated with various aminoglycosidic antibiotics (streptomycin, gentamycin, and neomycin) that were effective against *S. aureus*, *E. coli*, *P. aeruginosa*, and *Micrococcus luteus*.

Gold nanoparticle–carbohydrate conjugates can also be used to investigate multivalent carbohydrate–protein recognition processes. Lactose-biofunctionalized gold nanoparticles were found to bind a bivalent carbohydrate protein (lectin) of *Recinus communis* (Otsuka *et al.*, 2001). Similarly, Binding of mannose gold nanoparticles to Concanavalin A (Con A) and to type 1 fimbriae of living bacteria have also been reported (Lin *et al.*, 2002, 2003).

Gold nanoparticles containing galactosyl and glucosyl functional groups had a high affinity with recombinant HIV gp120 (Nolting *et al.*, 2003). Particles prepared from disulfides containing C-glycosides linked to triethylene glycol were 12% less active than biotinylated galactosyl ceraminde (GalCer), a water-soluble surrogate of GalCer. However, when these carbohydrates were presented on gold nanoparticles, they were 300 times more active than the disulfides and at least 20 times more active than biotinylated GalCer.

3. Copper nanomaterials

Copper nanoparticle/polymer composites have been evaluated as bioactive coatings with antifungal and bacteriostatic properties (Cioffi *et al.*, 2004, 2005). Cu nanoparticles prepared by electrochemical synthesis were embedded in polymer matrixes. These nanocomposites release levels of copper species capable of inhibiting *Saccharomyces cerevisiae*, *E. coli*, *S. aureus*, and *L. monocytogenes*. To minimize nanocomposite toxicity toward untargeted cells, release of Cu nanoparticles was controlled by dispersing a quantifiable amount of nanoparticles in the polymer matrixes. Esteban-Cubillo *et al.* (2006) embedded copper monodispersed nanoparticles (2–5 nm) into sepiolite (a magnesium phylosilicate). These Cu/Sepiolite particles achieved a reduction of 99.9% viability of *S. aureus* and *E. coli*.

B. Metal oxide nanomaterials

1. Titanium dioxide (TiO_2)

When illuminated with UV light, TiO_2 photocatalyst possesses strong oxidizing power by generating reactive oxygen species (ROS) such as hydroxyl radicals, hydrogen peroxide, and superoxide. These ROS exhibit excellent bactericidal activity (Lu *et al.*, 2003). A batch type photocatalytic reactor to investigate the bactericidal effects with various near-UV illumination times and TiO_2 concentrations was established (Kim *et al.*, 2003). When *S. choleraesuis*, *Vibro parahaemolyticus*, and *L. monocytogenes* were exposed to UV light for 2 hours, 5–20% of the cells lost viability while 95% of the bacteria lost viability when they were exposed under the same conditions with TiO_2. After 30 min of irradiation with UV light in the presence of TiO_2 at 1 mg/ml, 62% of *S. choleraesuis* and 80% of *V. parahaemolyticus* were killed. When the concentration of TiO_2 was decreased to 0.25 mg/ml, killing efficiency was decreased to 55% and 35%, respectively.

Amezaga-Madrid *et al.* (2003) tested the effects of TiO_2 thin films deposited on soda lime glass slides by sol–gel techniques. At 40 min, ~70% reduction of *P. aeruginosa* cells was observed. Kuhn *et al.* (2003) reported that bacteria placed on TiO_2 coated surfaces were killed in the decreasing order: *E. coli* > *P. aeruginosa* > *S. aureus* > *Enterobacter faecium* > *C. albicans*. While pure TiO_2 had no antibacterial effect in free solution or in agar, silver-doped TiO_2 particles were more effective than pure silver nanoparticles of similar size (Thiel *et al.*, 2007).

2. Magnesium oxide (MgO)

Stoimenov *et al.* (2002) prepared MgO via an aerogel procedure (AP-MgO), then adducted it with various halogens, resulting in nanoscale AP-MgO/X_2 (X = Cl, Br, or none). The ζ-potential (i.e., surface charge) of these nanoparticles was positive in aqueous suspension. Atomic force

microscopy (AFM) and TEM analysis of nanoparticles incubated with *E. coli*, *Bacillus megaterium* vegetative cells, and *B. subtilis* endospores revealed damage of cell membranes. AP-MgO penetration inside the cells resulted in the death of these bacteria (Fig. 4.7). The antimicrobial effects were attributed to the size, positive charge, high surface area, surface abrasiveness, and the active halogen of the AP-MgO/X_2. The positively charged particles bound to the negatively charged bacteria and spores. The intrinsic properties of small particle sizes provide a high surface area and enhanced surface reactivity. This allows many particles to cover the bacteria to bring a high concentration of active halogen into proximity with the bacterial surfaces.

MgO nanoparticles have excellent activity against *B. subtilis* spores and *S. aureus* (Huang *et al.*, 2005a,b). The active component of nano-MgO appears to be superoxide ions, which react with the peptide linkages in the cell wall of bacteria or spore. The antimicrobial activities are particle size-dependent (higher bactericidal activity with decreasing particle size). Nano-MgO has the advantage of being nontoxic to eukaryotic cells (Huang *et al.*, 2005a; Stoimenov *et al.*, 2002).

Makhluf *et al.* (2005) prepared MgO nanoparticles (5–12 nm) using a rapid microwave radiation procedure. MgO nanoparticle activity against *E. coli* and *S. aureus* was attributed to the small particle size and the

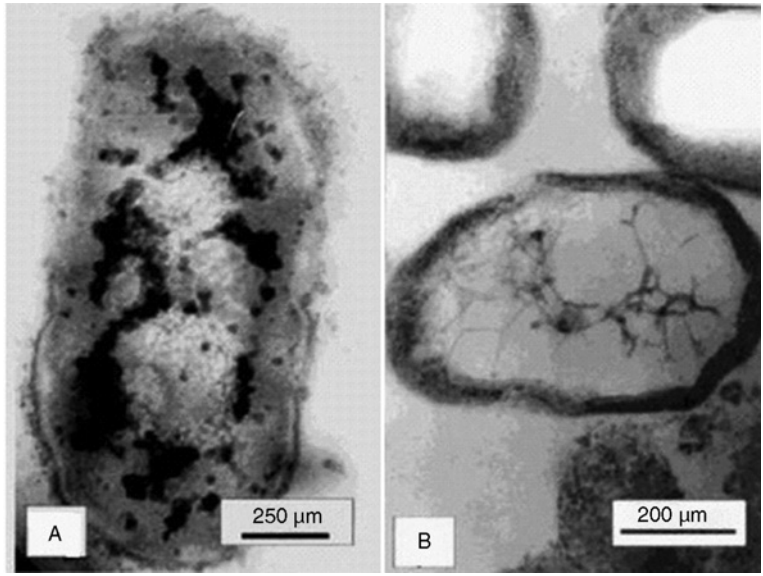

FIGURE 4.7 TEM micrographs of (A) the penetration of nanoparticles into *E. coli* and the damage of cell membrane, and (B) significant changes of *B. subtilis* spores upon AP-MgO/Cl_2 nanoparticles treatment. (Reproduced from Stoimenov *et al.*, 2002 with permission. Copyright © 2002 American Chemical Society.)

formation of an active oxygen species. Lin and Li (2005) dispersed MgO crystals at different loading amounts on a γ-Al_2O_3 support (MgO/γ-Al_2O_3). The activity of MgO/γ-Al_2O_3 against *B. subtilis* spores and *S. aureus* was attributed to the nano-MgO crystals formed on the outer surface of the support, not the γ-Al_2O_3 support itself. The threshold for bactericidal activity was 15% of maximal MgO loading. When MgO crystals were formed on the support, they killed more than 99% of *S. aureus.*

3. Zinc Oxide (ZnO)

Low concentrations of ZnO nanoparticles synthesized in diethylene glycol (DEG) medium by forced hydrolysis of ionic Zn^{2+} salts caused no apparent damage to *E. coli* cells. However, at concentrations $>1.3 \times 10^{-3}$ M, cell membranes were extensively damaged and disorganized, causing leakage of intracellular contents and cell death (Brayner *et al.*, 2006). The antibacterial activity of zinc oxide nanoparticles against *E. coli* was dependent on concentration and size. The antibacterial mechanisms were explained by direct interaction between ZnO nanoparticles and cell membrane components, based on evidence from electrochemical measurements and SEM analyses (Zhang *et al.*, 2007).

ZnO nanostructures have also received considerable attention because of their desirable optical properties, which are useful in augmenting the fluorescence signal from fluorophore-linked DNA molecules and promoting detection at ultra-trace concentration. ZnO nanomaterials can serve as excellent signal-enhancing substrates for hybridization reactions of model DNA systems of genetically related *Bacillus*. Both covalent and noncovalent linking schemes have been used to couple probe DNA strands to ZnO nanostructures (Kumar *et al.*, 2006). These ZnO nanostructures can be effectively used for the identification of *B. anthracis* by discriminating its DNA sequence from other genetically related species. The use of ZnO nanomaterials greatly enhances the fluorescence signal collected from duplex formation reactions. The covalent strategy allows detection of the target species at femtomolar sample concentrations, when compared with the detection sensitivity in the nanomolar range when using the noncovalent method. The presence of the underlying zinc oxide nanomaterials is critical in achieving increased fluorescence for detection of hybridized DNA and, therefore, accomplishing rapid and sensitive identification of a biothreat.

C. Magnetic nanomaterials for the detection of microbes

1. Immunomagnetic detection

Immunomagnetic nanomaterial probes have been developed to detect or separate *E. coli* O157:H7 from mixed cultures. Varshney *et al.* (2005) prepared immunomagnetic nanoparticles by immobilizing biotin-labeled polyclonal

goat anti-*E. coli* antibodies on streptavidin-coated magnetic nanoparticle complex (MNCs). These MNCs were mixed with ground beef samples inoculated with *E. coli* O157:H7. Nanoparticle–antibody-*E. coli* O157:H7 aggregates were separated from food samples with an external magnet. A minimum capture efficiency of 94% was achieved by MNCs for *E. coli* O157:H7 ranging from 1.6×10^1 to 7.2×10^7 CFU/ml without any enrichment. The capture has been shown to be very specific without any interference from *Salmonella enteritidis, Citrobacter freundii*, and *L. monocytogenes*.

Immuno-nanotubes can also capture pathogens via immunomagnetic separation (Lin *et al.*, 2006). Bovine serum albumin (BSA)-biofunctionalized MWNTs with encapsulated ferromagnetic elements (magnetic-MWNTs-BSA) were conjugated with goat anti-*E. coli* O157:H7 antibody to form ''immunomagnetic-MWNTs.'' These immunomagnetic-MWNTs captured *E. coli* O157:H7 from aqueous suspension as aggregates, which are visible under scanning electron microscopy (Fig. 4.8). The immunomagnetic-MWNTs captured *E. coli* O157:H7 with high sensitivity (4×10^2 CFU/ml in pure culture) and selectivity (no background *S. typhimurium* interference from mixed culture).

Magnetic iron-dextran nanoparticles, coated with anti-*E. coli* O157:H7 IgG by the periodate oxidation-borohydride reduction procedure, had a detection sensitivity limit of 10 CFU/ml within a 15 min reaction time (Duan *et al.*, 2005). For detection of *E. coli* O157:H7 in mixed culture (10^8 CFU/ml of other organisms), a sensitivity of 10^1–10^2 CFU/ml could be achieved. Nonspecific binding of other bacteria was not observed. An enrichment time of 2.5 hours was enough for the particles to detect targeted bacteria in food samples inoculated with 1 CFU/g.

Covalent attachment of IgG to surfaces of Fe_3O_4 magnetic nanoparticles (Fe-IgG) generated biofunctionalized affinity probes for selectively concentrating traces of *S. aureus* and *Staphylococcus saprophyticus* from sample solutions (Ho *et al.*, 2004). The nanoparticle-trapped bacteria were then separated from solution by an external magnet and subsequently characterized by matrix-assisted laser desorption/ionization mass spectrometry (MALDI–MS).

2. Antibiotic magnetic nanoparticles

Vancomycin (Van) binds to a terminal peptide D-Ala-D-Ala on the bacterial cell wall via hydrogen bonding. Vancomycin-bearing magnetic FePt nanoparticles offer promise for instant and ultrasensitive pathogen detection (Gu and Xu, 2006). Van-modified FePt nanoparticles were able to detect Gram-negative and Gram-positive (Gu *et al.*, 2003b, 2003c) bacteria at concentrations of less than 10^2 CFU/ml within an hour. After mixing biofunctional magnetic nanoparticles with a suspension of *Enterococci*, coliforms, and *Staphylococci* for 20 min, an external magnet was employed to separate the aggregates of nanoparticles and bacteria as verified by SEM.

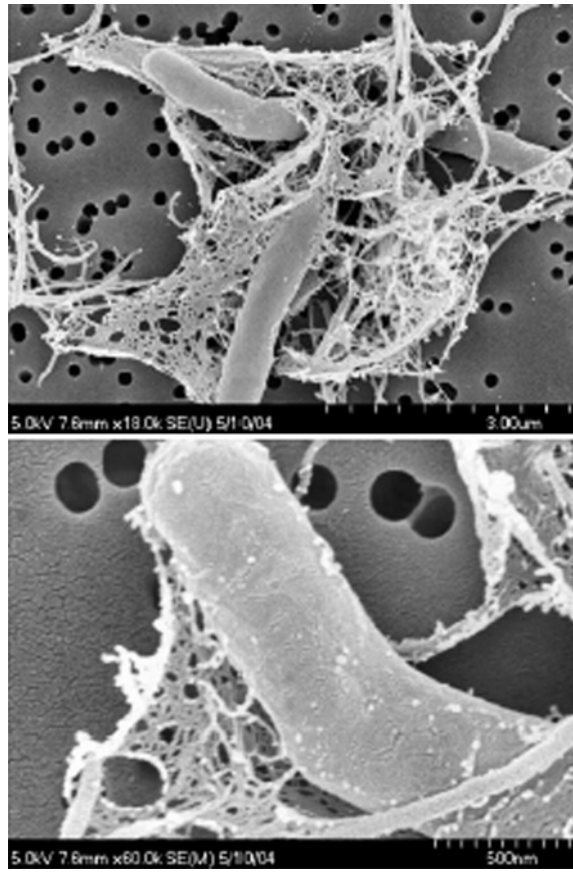

FIGURE 4.8 SEM images of the *E. coli* cells bound with immunomagnetic-MWNTs. (Reproduced from Lin *et al.*, 2006 with permission. Copyright © 2006 American Scientific Publishers.)

Because different species of the same bacterial genus share similar morphologies, they cannot be easily differentiated after separation from complex biological fluids by antibiotic magnetic nanoparticles affinity probes. However, MALDI–MS was effective in differentiating microbial species in magnetic nanoparticle aggregates based on their mass spectra (Lin *et al.*, 2005).

V. CONCLUDING REMARKS

Nanoparticles and nanotubes have been generated from a variety of bulk organic and inorganic materials that provide a versatile array of antimicrobial mechanisms. Antiadhesion mechanisms target the first step of the

microbial infection process and therefore hold great promise (Karlsson, 1999; Luo *et al.*, 2005a; Sharon and Lis, 1989, 1993). Through specific binding, sugar-biofunctionalized nanomaterials compete with host cells for the microbial surface protein receptors (lectin-type adhesins) to interfere with microbial colonization. For successful clinical use of adhesin-specific nanomaterials as therapeutic agents, nanoparticles must have certain characteristics. The ''Ten Commandments'' for an ideal clinical antiadhesion nanomaterial might read something like this from the microbiologists' viewpoint:

1. Nanoparticles must be of low toxicity to the host (high host toxicity renders all other characteristics meaningless).
2. Nanoparticles must not be immunogenic (nanoparticle immunogenicity would limit therapeutic effectiveness in continued treatment or trigger hypersensitive reactions in the host).
3. The target of nanoparticle activity must be specific (if the nanoparticle target is exhibited by normal host flora as well as by the pathogen, therapeutic effectiveness would be compromised).
4. The nanoparticle target site must be surface-bound (spikes, envelope, or capsid subunits in the case of viruses, or adhesins in the case of bacteria).
5. Nanoparticle activity should be directed toward an essential virulence factor (mutation to nanoparticle resistance would require loss of the virulence factor or change in its conformation, thereby lowering pathogenicity).
6. Nanoparticles should be resistant to degradation by microbial or host enzymes (nanoparticle degradation would destroy activity or release toxic by-products).
7. Nanoparticles must not be inactivated by other medications or antagonistic to their action (drug antagonisms are common hazards in clinical use).
8. The synthetic platform for nanoparticle production must be versatile (the basic particle must be able to act as vehicle for a variety of different biofunctional groups).
9. Nanoparticles must be amenable to mass production (economy demands large scale production using conventional industrial equipment).
10. Nanoparticles must have long shelf life (chemical stability is particularly important in developing countries where storage facilities are inadequate or unavailable).

Because carbohydrates play important roles in adhesion in other physiological and pathological processes, such as inflammation and metastasis (Varki, 1993, 1994), it is not surprising that studies have been undertaken using the antiadhesion strategy to interfere with these

processes. Gold glyconanoparticles have already been applied as antiadhesion tools against progression of metastasis (Rojo *et al.*, 2004). The Ten Commandments for antimicrobial adhesion could also be applied to the antiadhesion of inflammatory and cancer cells.

Other antimicrobial strategies include multivalency, innate killing properties, photocatalytical capability of metal oxide nanomaterials, vaccination, and photothermal therapeutics. Multivalency could be utilized to generate enhanced activity (e.g., of antibiotics, carbohydrates) (Gu *et al.*, 2003a; Nolting *et al.*, 2003; Youssef *et al.*, 1988). The well-developed chemistry of nanomaterials provides freedom to incorporate a variety of biomolecules (carbohydrates, antibiotics, peptides, proteins) on the surface of nanomaterials. Antiadhesion therapy and multivalency take advantage of the specificity between receptor and ligands (i.e., carbohydrate and antibiotic), as well as multivalent interaction. A third method uses the broad antimicrobial properties of silver (Aymonier *et al.*, 2002; Jiang *et al.*, 2004; Lok *et al.*, 2006) and chitosan (Qi *et al.*, 2004; Shi *et al.*, 2006). Attempts have also been made to combine two different microbicidal mechanisms to achieve higher efficiency. For instance, silver nanoparticles in combination with amoxicillin showed a synergistic antibacterial effect against *E. coli* (Li *et al.*, 2005). A fourth method makes use of the inherent properties of certain metallic oxide nanomaterials for the antimicrobial effects via the photocatalytic capability of TiO_2 to generate hydroxyl radicals (Brayner *et al.*, 2006; Kim *et al.*, 2003; Kuhn *et al.*, 2003; Stoimenov *et al.*, 2002). A fifth method is the development of nanomaterials as vaccine carriers, adjuvants, or even vaccine themselves (Baudner *et al.*, 2002; Kim *et al.*, 1999; Raman *et al.*, 2005). Finally, photothermal therapeutic use of light-absorbing gold nanoparticles for the selective killing of bacteria is promising (Naruse, 2005; Zharov *et al.*, 2006).

Nanomaterials bioconjugated with proteins (including antibodies), antibiotics, carbohydrates, or nucleic acids have also been explored as probes to separate or to identify various microbes. While luminescent nanoparticles (e.g., QDs) can be used in fluorescent detection, most of the detection/separation strategies take advantage of the specificity of biological interactions. These pairwise interactions include antigen–antibody (Duan *et al.*, 2005; Varshney *et al.*, 2005; Zhao *et al.*, 2004a), antibioti–protein (Gu *et al.*, 2003a,b,c), carbohydrate–adhesin (Luo *et al.*, 2005b, 2006), DNA–DNA (complementary sequences) (Wabuyele and Vo-Dinh, 2005), and biotin–avidin (Edgar *et al.*, 2006; Varshney *et al.*, 2005). The use of nanotechnology in pathogen isolation and detection provides greater sensitivity, potential for lower cost, shorter turn-around time, smaller sample size, in-line and real-time detection, higher throughput, portability for in-field applications, surface decontamination mechanisms, as well as applications in drug discovery, food safety, and homeland security.

A major concern in the clinical use of nanomaterials is their potential health risk. Although some nanomaterials have been shown to be toxic (Hardman, 2006; Lam *et al.*, 2004), most nanomaterials that are tested for antimicrobial applications showed no apparent or little toxicity in *in vitro* and *in vivo* studies (Franklin *et al.*, 2003; Huang *et al.*, 2005a; Molugu *et al.*, 2006; Shi *et al.*, 2006). The next phase of nanomaterial research must include clinical studies using animal models to assess the toxicity potential of these intriguing and versatile bioagents.

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CHAPTER 5

Metabolic Aspects of Aerobic Obligate Methanotrophy*

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* This review is dedicated to the memory of Professor J.R. Quayle, FRS (1926–2006), who discovered many of the unique primary pathways of carbon metabolism in aerobic methanotrophs and methylotrophs

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I. INTRODUCTION

Aerobic methanotrophs are a unique group of gram-negative bacteria that use methane as carbon and energy source and were first described by Kaserer (1905, 1906) and by Söhngen (1906, 1910). Methanotrophs have been studied intensively over the past 40 years since these bacteria possess significant metabolic potential for practical use in the biotransformation of a variety of organic substrates, bioremediation of pollutants (e.g., halogenated hydrocarbons), the production of single-cell protein (SCP), and value-added products (e.g., epoxypropane from propene). They also play a vital role in the global methane cycle, mitigating the emissions and green-house effects of methane on the Earth's climate. Methanotrophs build all of their cell constituents from C_1 compounds by employing special biosynthetic pathways for phosphotrioses, which are different from those of heterotrophic bacteria. Metabolic specialization of methanotrophs related to their utilization of CH_4, a highly reduced gaseous substrate, is reflected in the ultrastructure of methanotroph cells, which possess well-developed intracytoplasmic membranes (ICM) arranged either as bundles of vesicular disks distributed throughout the cell (type I methanotrophs) or as paired peripheral layers (type II methanotrophs). These two major types of ICM arrangement correlate with the Quayle ribulose monophosphate (RuMP) and serine cyclic pathways of C_1 assimilation, respectively (Lawrence and Quayle, 1970; Shishkina *et al*., 1976). Possible reasons for such a structure–functional correlation might be due to the different efficiency of energy-yielding processes occurring in the type I and type II ICM as well as various energy demands/requirements of the RuMP and serine pathways (Leak *et al*., 1985). It is not possible to cover all aspects of the biology of methanotrophs in this review, so we refer the reader to the comprehensive publications and monographs related to taxonomy, ecophysiology, and biotechnological applications of obligate aerobic methanotrophs (Anthony, 1982, 1991; Dalton, 2005; Hanson and Hanson, 1996; Large and Bamforth, 1988;

Murrell and Dalton, 1992; Smith and Dalton, 2004). In this review we will focus on the metabolic and genetic organization of these unique bacteria, particularly with respect to their carbon and nitrogen metabolism, because of the limited number of recent reviews on this subject available in the literature.

II. MILESTONES IN AEROBIC OBLIGATE METHANOTROPHY: A BRIEF HISTORICAL OVERVIEW

A. Discovery of aerobic methanotrophs and first impacts on methanotrophy

This review has been written for two reasons. Firstly, it is just over 100 years since the first scientific reports on the isolation of methane-oxidizing (methanotrophic) bacteria (Kaserer, 1905, 1906; Sohngen, 1906, 1910). Secondly, there has been a steady increase in the biology of these unique microorganisms because they contribute significantly to the global balance of atmospheric methane, estimated to be 25 times more effective as a greenhouse gas than CO_2. Evidently, the discoverers of methanotrophic bacteria, Kaserer and Söhngen, could have scarcely imagined the importance of these bacteria or their ubiquity in nature, apart from other scientific and practical consequences of their discovery. Furthermore, few microbiologists followed up the discovery of methanotrophs over the next 50 years and the cultures of methanotrophs were apparently lost. Fundamental work by microbiologists and biochemists, primarily in the UK and the USA, gave renewed impetus to our understanding of the biology of methanotrophs. At the beginning of the 1970s, the next generation of scientists were to address a number of basic questions including the following:

- What is the taxonomic diversity of methanotrophs and the distribution of methanotrophy in the microbial world?
- How do methanotrophs oxidize methane and obtain energy for phosphotriose synthesis?
- What are the pathways of primary and intermediate metabolism of carbon and nitrogen?
- What is the genetic organization of the biosynthetic and energy-yielding pathways of methanotrophs?
- What is the biogeochemical role that methanotrophs play in the global cycles of carbon and nitrogen?
- What contribution do methanotrophs make to the cycling of methane in the biosphere?
- What microorganisms are responsible for anaerobic oxidation of methane?
- What is the biotechnological potential of methanotrophs?

The majority of these questions have been answered because of the pioneer work outlined in Table 5.1, which lists the milestones in the history of research on methanotrophs.

TABLE 5.1 Milestones in the Study of Obligate Methanotrophs

I. *Biodiversity of aerobic methanotrophs*

1. Discovery of the process of bacterial methane oxidation (Kaserer, 1905, 1906; Söhngen, 1906, 1910).
2. Isolation of the first monocultures of *Pseudomonas (Bacillus) methanica* (Söhngen, 1906), *Methylomonas methanica* (Dworkin and Foster, 1956), *Methylococcus capsulatus* (Foster and Davis, 1966), and *Methanomonas methanooxidans* (Brown *et al*., 1964).
3. Creation (formation) of collections and taxonomic frameworks for obligate methanotrophs (Bowman *et al*., 1993, 1995; Malashenko, 1976; Trotsenko, 1976; Whittenbury, 1969; Whittenbury *et al*., 1970).
4. Isolation and characterization of extremophilic methanotrophs: thermophiles (Bodrossy *et al*., 1995, 1997, 1999; Malashenko, 1976; Tsubota *et al*., 2005), psychrophiles (Bowman *et al*., 1997; Omelchenko *et al*., 1993, 1996), haloalkalophiles (Heyer *et al*., 2005; Khmelenina *et al*., 1997; Lidstrom, 1988; Sorokin *et al*., 2000), and acidophiles (Dedysh *et al*., 2000, 2002, 2005; Dunfield *et al*., 2007; Islam *et al*., 2008; Pol *et al*., 2007).

II. *Enzymology of pathways of methane oxidation*

1. Discovery of methane oxygenation (Higgins and Quayle, 1970). Purification and characterization of sMMO (Colby *et al*., 1977; Tonge *et al*., 1977) and pMMO (Basu *et al*., 2003; Lieberman *et al*., 2003; Nguyen *et al*., 1998), mechanisms of their action, regulation and expression (reviewed by Dalton, 2000).
2. Isolation and characterization of the gene cluster *mmoX,Y,B,Z,D,C* encoding sMMO proteins (reviewed in Murrell *et al*., 2000a,b). Molecular analysis and regulation of the MMO gene cluster in *Mc. capsulatus* Bath and *Ms trichosporium* OB3b (Nielsen *et al*., 1996; Stainthorpe *et al*., 1989, 1990a). Design of specific functional gene probes *mmo*X, *pmo*A, and *mxa*F for methanotrophs (McDonald *et al*., 1995).
3. Discovery of the H_4MPT pathway enzymes in methanotrophs (Vorholt *et al*., 1999).

III. *Alternative pathways of primary C_1-assimilation in methanotrophs*

1. Discovery and elucidation of the RuMP and serine cycles in type I and type II methanotrophs (Quayle, 1969, 1972, 1980).
2. Discovery of the enzymes and genes of the minor RuMP and serine pathways and ribulose bisphosphate carboxylase/oxygenase in

(*continued*)

Table 5.1 (*continued*)

type X methanotrophs *Methylococcus* (Stanley and Dalton, 1982; Taylor, 1977, 1981) and *Methylocaldum* (Baxter *et al*., 2002; Bodrossy *et al*., 1997).

3. Evidence for operation of the novel variant of the RuMP cycle coupled with PP_i-6-phosphofructokinase (Trotsenko and Shishkina, 1990; Trotsenko *et al*., 1996).

IV. *Pathways of intermediate metabolism in obligate methanotrophs*

1. Dichotomy of the pathways of C and N metabolism (Davey *et al*., 1972; Shishkina and Trotsenko, 1979; Shishkina *et al*., 1976).
2. Characterization of the enzymes and genes involved in N_2 fixation (Murrell and Dalton, 1983a,b,c).
3. Finding of multiple enzymic lesions in central metabolic pathways (tricarboxylic acid cycle, glyoxylate bypass, glycolysis, and gluconeogenesis) of obligate methanotrophs (Davey *et al*., 1972; Shishkina and Trotsenko, 1982; Trotsenko, 1983).

V. *Functional genomics and proteomics of obligate methanotrophs*

Sequencing of total genomes of *Methylomonas* sp. 16A (Sharpe *et al*., 2002) and *Mc. capsulatus* Bath (Kelly *et al*., 2005; Ward *et al*., 2004). First proteomic studies on *Mc.capsulatus* Bath (Berven *et al*., 2006; Kao *et al*., 2004)

VI. *Ecophysiology and biotechnological potential of obligate methanotrophs*

1. Evidence for global biogeochemical role of methanotrophs in C and N cycles (reviewed in Hanson and Hanson, 1996).
2. Discovery of methanotrophic symbionts in marine invertebrates (Cavanaugh *et al*., 1987; Childress *et al*., 1986) and plants (Raghoebarsing *et al*., 2005; Trotsenko *et al*., 2001).
3. Using methanotrophs for biodegradation of halogenated hydrocarbons, production of SCP, commodity, and fine chemicals, for example, epoxypropane from propene (reviewed in Dalton, 2005; Hanson and Hanson, 1996; Large and Bamforth, 1988; Smith and Dalton, 2004) enzymes (Yu *et al*., 2003), and the synthesis of ectoine (Trotsenko *et al*., 2005).

VII. *Discovery of facultative methanotrophs*

1. The first fully authenticated facultative methanotroph *Methylocella silvestris* BL2 (Dedysh *et al*., 2005, Theisen *et al*., 2005; reviewed in Theisen and Murrell, 2005).
2. The novel filamentous facultative methanotrophs *Crenothrix polyspora* (Stoecker *et al*., 2006) with an unusual methane monooxygenase (*pmoA*) and *Clonothrix fusca* (Vigliotta *et al*., 2007).

The 1970s certainly saw considerable research activity aimed at realizing the biotechnological potential of methanotrophs. It was shown that methanotrophic bacteria are of significant biotechnological interest since they can be used in the production of single-cell protein (SCP) for animal feed, enzymes, lipids, sterols, antioxidants, pigments, polysaccharides, factors that promote iron transport, primary and secondary metabolites (amino acids, organic acids, solvents, vitamins, alkaloids, antibiotics), biotransformation of organic compounds, control of methane in coal mines, and design of biosensors and fuel generating processes (reviewed in Higgins *et al.*, 1980, 1981; Large and Bamforth, 1988). However, after the 1980s there was loss of interest in the commercial production of SCP from methane, partly due to the lack of appropriate molecular genetics methods for methanotrophs. This included difficulties in obtaining mutants and their subsequent genetic analysis, which called for gaining further insights into the biology and regulation of methane oxidation.

B. Renaissance of interest in the biology and biochemistry of methanotrophs

Recognition of the hazards posed by changes in global climate, which will probably occur in the next 100 years or so as a consequence of the decrease in global vegetation and contamination of the biosphere by anthropogenic activity, has prompted ecologists and biogeochemists to study the contribution of methanogens and methanotrophs to global warming. Their roles in controlling the methane cycle on the Earth are undoubtedly significant. However, the annual increase in the atmospheric methane concentration is around 1%, due to the imbalance between the biological and anthropogenic production of methane and its degradation in the environment (Hanson and Hanson, 1996; Reeburgh *et al.*, 1993).

In the last two decades, study of the ecology of methanotrophs and the biogeochemistry of the methane cycle has intensified as a result of improved methods of *in situ* quantification of methanotrophs and their activity. These advances include techniques for radioisotopic measurements of the rates of methane production and oxidation as well as analysis of the stable isotopic composition of methane in the biosphere.

Over the last two decades, interest in the phenomenon of methanotrophy has been rekindled owing to the application of molecular microbial methods for the study of qualitative and quantitative composition of methanotrophic communities (reviewed in Dumont and Murrell, 2005; Murrell *et al.*, 1998). This also occurred when extremophilic and extremotolerant methanotrophs were discovered (Trotsenko and Khmelenina, 2002) and the taxonomic status of well-known and new species of methanotrophs was refined by molecular biology methods.

The Earth's biosphere has a number of extreme physicochemical environments and methanotrophs that exist in such conditions (especially if the relatively low solubilities of CH_4 and O_2 are taken into account) have attracted the particular attention of microbiologists. However, only in the last decade have investigations of ecosystems with high and low values of pH, temperature, or salinity led to the discovery of several new physiological subgroups of extremophilic and extremotolerant methanotrophs (Trotsenko and Khmelenina, 2002; Table 5.2).

In addition to the well-known thermophilic and thermotolerant species of the genera *Methylococcus*, *Methylocaldum,* and *Methylothermus* (Bodrossy *et al*., 1995, 1997, 1999; Malashenko, 1976), a new species of moderately thermophilic methanotrophs has been described—*Methylothermus thermalis*, which grows at a temperature level up to 65 °C (Tsubota *et al*., 2005). However, the molecular mechanisms of thermoadaptation in these methanotrophs are still unknown.

TABLE 5.2 Multiple Enzymic Lesions in Central Metabolism of Obligate Methanotrophs

	Methanotrophs		
Enzyme	Type I	Type II	Type X
Hexokinase	+	−	−
6-Phosphofructokinase			
ATP	−	−	−
PP_i	+	+	+
FBP-aldolase	+	−	+
KDPG-aldolase	+	−	+
Glucose-6-phosphate dehydrogenase	+	−	+
Fructose-1,6-bisphosphatase	−	−	−
Sedoheptulose-1,7-bisphosphatase	−	−	−
Pyruvate kinase	−	−	−
Pyruvate dehydrogenase	+	−	+
α-Ketoglutarate dehydrogenase	−	+	−
Isocitrate lyase	−	−	−
Malate synthase	−	−	−
PEP-synthase	−	−	−
PEP-carboxykinase	−	−	−
PEP-carboxylase	+	+	(+)
PEP-carboxytransphosphorylase	−	−	−
Pyruvate-phosphate dikinase	−	−	−
Pyruvate carboxylase	+	−	−

Abbreviations: FBP, fructose-1,6-bisphosphate; KDPG, 2-keto-3-deoxy-6-phosphogluconate; PEP, phospho-(enol)pyruvate.

The optimal growth temperature of *Methylobacter psychrophilus*, the first psychrophilic methanotroph isolated (Omelchenko *et al*., 1993, 1996), is 5–10 °C, but it cannot grow at a temperature above 20 °C. Activity of most enzymes of primary and central metabolic pathways decreased markedly as the growth temperature drops from 20 °C to 5 °C. However, the structural and functional mechanisms of adaptation of this organism as well as other psychrophilic methanotrophs (e.g., *Methylosphaera hansonii*) remain unknown (Bowman *et al*., 1997; Trotsenko and Khmelenina, 2005).

Acidophilic and acidotolerant methanotrophs also inhabit the psychrosphere, which includes extensive wetlands in the Northern Hemisphere and Western Siberia. These methanotrophs are represented by new species of the genera *Methylocella* and *Methylocapsa,* which optimally grow at a pH of 4.5–5.5 and differ substantially from other methanotrophs in the morphology and ultrastructure of their vegetative and resting cells (Dedysh *et al*., 2000, 2002, 2005; Dunfield *et al*., 2003; Table 5.3).

The observation that *Methylocella palustris* lacks well-developed ICM and a membrane-associated MMO (see later) as well as the capacity of the *Methylocella* species for heterotrophic growth is quite surprising (Dedysh *et al*., 2005; Theisen and Murrell, 2005; Theisen *et al*., 2005). Evidently, these methanotrophs form two distinctive geno- and phenotypes. The ability to grow at low salinity, as well as the sensitivity to inhibition by some cations, which distinguish them from haloalkalophilic methanotrophs, is striking. The structural and functional organization of these bacteria as well as their molecular mechanisms of acidotolerance are poorly understood.

The most well-known haloalkalophilic and haloalkalotolerant methanotrophs, tentatively assigned to the genus *Methylomicrobium*, use two main strategies of adaptation to increased salinity of the environment and survive at NaCl concentrations of 12% (w/v) and at a pH of 9–11. One such survival strategy of these bacteria involves the *de novo* synthesis and intracellular accumulation of organic osmoprotectants (ectoine, glutamate, and sucrose) as well as K^+ ions. Other adaptive strategies involve some structural and functional changes in cell envelopes, namely, rearrangements in the phospholipids composition of membranes and formation of surface glycoprotein S-layers. As to the bioenergetics and molecular regulation of osmoadaptation of aerobic methanotrophs, only preliminary data are available (Khmelenina *et al*., 1997, 1999). Moreover, nothing is known about osmoadaptation of mesophilic and neutrophilic type I methanotrophs, tolerant of up to 15% NaCl (w/v), which have recently been assigned to the novel taxon *Methylohalobius crimeensis* (Heyer *et al*., 2005).

Overall, the recently isolated extremophilic/extremotolerant methanotrophs will provide interesting models for studying the mechanisms of adaptation to various extreme conditions. These investigations will shed

TABLE 5.3 Current Classification of Aerobic Methanotrophs

GAMMAPROTEOBACTERIA Order: Methylococcales Family: Methylococcaceae			ALPHAPROTEOBACTERIA Order: Rhizobiales Family: Methylocystaceae
Type I		Type X	Type II
Methylomonas	***Methylobacter***	***Methylococcus***	***Methylocystis***
Methylomonas methanica	*Methylobacter bovis*	*Methylococcus capsulatus*	*Methylocystis parva*
Methylomonas fodinarum	*Methylobacter chroococcum*	*Methylococcus thermophilus*	*Methylocystis echinoides*
Methylomonas aurantiaca	*Methylobacter vinelandii*		*Methylocystis rosea*
Methylomonas rubra	*Methylobacter psychrophilus*	***Methylocaldum***	*Methylocystis pyriformis*
Methylomonas scandinavica	*Methylobacter luteus*	*Meth1ylocaldum gracile*	*Methylocystis methanolicus*
	Methylobacter tundripaludum	*Methylocaldum szegediense*	*Methylocystis minimus*
	Methylobacter marinus	*Methylocaldum tepidum*	*Methylocystis heyeri*
Methylomicrobium			*Methylocystis hirsuta*
Methylomicrobium pelagicum	***Methylosphaera***		
Methylomicrobium album	*Methylosphaera hansonii*		***Methylosinus***
Methylomicrobium agile			*Methylosinus trichosporium*
Methylomicrobium buryatense	***Methylosarcina***		*Methylosinus sporium*
	Methylosarcina fibrata		
Methylohalobius	*Methylosarcina quisquilibrium*		Family: Beijerinckiaceae
Methylohalobius crimeensis	*Methylosarcina lacus*		
			Methylocella
Crenothrix	***Methylothermus***		*Methylocella palustris*
Crenothrix polyspora	*Methylothermus thermalis*		*Methylocella tundrae*
			Methylocella silvestris
Clonothrix			
Clonothrix fusca			***Methylocapsa***
			Methylocapsa acidiphila

more light on the biology of aerobic methanotrophs and answer the main question of whether these bacteria have specific molecular mechanisms, that is, receptor and stress-responding proteins, polypeptides, signals and factors, or do they use the mechanisms found in other prokaryotes. New approaches are needed to understand the molecular mechanisms of their adaptation to various stresses, in particular, those that provide the highest and lowest temperature necessary for growth and responses to external pH and salinity. It is highly plausible that comparative genomics and proteomics will help us to understand the molecular basis of survival as well as the evolution and phylogeny of various extremophilic and extremotolerant aerobic methanotrophs with respect to other prokaryotes. Extremophilic and extremotolerant methanotrophs have the potential for the production of stable (robust) enzymes (extremozymes) and bioprotectants, such as ectoine, resulting in new possibilities for the use of these methanotrophs in biotechnology. Both these methanotrophs are presumably more important for the global ecology and environmental bioremediation, owing to their specific metabolic potential and capacity for the aerobic degradation of different natural pollutants under extreme conditions (Murrell *et al*., 1998; Trotsenko and Khmelenina, 2002, 2005).

Extremophilic and extremotolerant methanotrophs are of immediate interest as models for astrobiology, since methane, methanol, and formaldehyde, as components of the terrestrial and cosmic cryolithosphere (cryptosphere), could have been able to assist the formation and distribution of extra-terrestrial life forms, including anaerobic and aerobic methanotrophs. It has been suggested that methanotrophs can hold a certain position in the hypothetical cryptobiosphere of Mars (Quayle and Ferenci, 1978). The detection of genes and enzymes of the tetrahydromethanopterin (H_4MPT) pathway of formaldehyde oxidation in methylotrophs and methanotrophs is an argument in support of the parallel emergence and development of these two forms of methylotrophic microbiota (Vorholt, 2002). In addition, the genes encoding the key enzymes of the RuMP cycle, hexulosephosphate synthase (*hps*) and phosphohexuloisomerase (*hpi*), have also been found in some methane producing Archaea whose pentose phosphate pathway is imperfect (Kato *et al*., 2006; Mitsui *et al*., 2000).

C. New findings (insights) in methanotrophy assessed by molecular approaches

It has been shown that copper plays a central role in the metabolism of methanotrophs, exerting control over the expression of soluble methane monooxygenase (sMMO) and particulate methane monooxygenase

(pMMO) (see Section III), formaldehyde dehydrogenase, and a number of polypeptides associated with the regulation and transport of copper ions; however, the nature of the transporters (siderophores) involved was unknown. Deciphering the molecular structure of methanobactin, a novel chromopeptide (1.2 kDa) which many methanotrophs release into the medium and which may be a transporter of copper ions and hence important in copper homeostasis, is a recent achievement (Kim *et al*., 2004, 2005). Methanobactin has a very compact pyramid-like shape with the metal complexation site located at the base of the pyramid. Whether methanobactin acts exclusively as an extracellular copper sequestering agent, or has other *in vivo* functions related to the delivery and insertion of Cu ions to pMMO, must be still determined. Methanobactin, being primarily a putative ''copper shuttle,'' is functionally and structurally close to the pyoverdin class of iron siderophores, for example, azotobactin and pseudobactin produced by *Azotobacter* and *Pseudomonas,* respectively. In contrast to iron siderophores, methanobactin binds a variety of metals. Hence methanotrophs may play a role in solubilization and mobilization of many metals *in situ*. This ability of methanotrophs can be used for removing and transferring radionuclides and heavy metals from hazardous waste sites to surrounding areas (Choi *et al*., 2006). Also, methanobactin has an unusual thiopeptide bond previously found in the nickel methyl reductase of methanogenic Archaea, which catalyzes CH_4 production from methyl coenzyme M and coenzyme B (Crabarse *et al*., 2000; Kim *et al*., 2004). This further supports the suggestion that methanotrophs may be more closely related to methanogens on an evolutionary scale than thought earlier.

Lastly, it is important to draw attention to the prediction of gene functions in the newly sequenced genome of *Methylococcus capsulatus* Bath (Ward *et al*., 2004), which appears to possess three pathways of primary C_1 assimilation and has thus been termed a type X methanotroph having a ''metabolic mosaic'' (Whittenbury, 1980). Undoubtedly, the availability of genome sequence information is of profound importance for the development of molecular microbiology of aerobic methanotrophs and can be considered as an outstanding achievement and a new milestone, which will provide additional insights into whether the genomic potential of methanotrophs is consistent with their physiological and biochemical activity. A platform by which we can use bioinformatics, marker exchange mutagenesis, and proteomics to investigate methanotrophs (including this one) has now been created. A detailed genome analysis, which is in a sense a ''road-map,'' may be helpful in studies of the molecular organization and metabolic regulation in obligate and facultative methanotrophs. In particular, this might explain the inability of these bacteria to grow on substrates other than methane and methanol, such as multicarbon compounds (see later).

Ward *et al*. (2004) have made a substantial annotation of 3113 open reading frames (ORFs) that are encoded by the 3.3 Mb genome of *Mc. capsulatus* Bath (reviewed by Kelly *et al*., 2005). Since, this genome sequence encodes as many as 1000 hypothetical proteins, the potential for finding new metabolic features is indeed very big. For example, the genome analysis has widened a previously described new class of the cytochrome P450 superfamily in *Mc.capsulatus* Bath from three to six members. The same 25-kb region contains novel proteins related to energy metabolism (c-type cytochromes and their biogenesis proteins, flavodoxin domain, proteins involved in heme transport and Fe-S binding) (Bergmann *et al*., 1999). The redundancy in cytochrome c_{553} proteins suggests a more complex and plastic electron transport chain capability for this methanotroph (Ward *et al*., 2004).

The most important questions that have to be answered yet are probably the following: what are the exact mechanisms of MMO regulation by copper ions, what are the roles of the numerous organic and inorganic transporters in the genomes of these bacteria, what is the molecular basis of obligate methanotrophy (recently reviewed by Wood *et al*., 2004). Evidently, the genome sequence information needs to be used in conjunction with carefully defined physiological experimentation and recently developed tools in molecular genetics of methanotrophs, in order to tackle these questions and to be able to improve our understanding of the molecular mechanisms that control the biology of aerobic methanotrophs.

Now, we briefly mention a sensational discovery of methane-utilizing ability in two well-known filamentous sheathed microorganisms belonging to Gammaproteobacteria—*Crenothrix polyspora* (Stoecker *et al*., 2006) and *Clonothrix fusca* (Vigliotta *et al*., 2007). This was supported by methane uptake and assimilation experiments, the occurrence of the type I ICM, and several *pmoA* genes. Strikingly, *C. polyspora* possessed a phylogenetically unusual *pmoA,* whose expression was strongly increased in the presence of methane. These recent findings clearly demonstrated new horizons in unravelling the complexity of structure–function organization and evolution of methanotrophs.

III. PATHWAYS OF SEQUENTIAL OXIDATION OF METHANE TO CO_2

A. Enzymes of primary methane oxidation

It is generally accepted that methanotrophs oxidize methane to carbon dioxide and water via the intermediates methanol, formaldehyde, and formate (Fig. 5.2). Initial oxidative attack of CH_4 is catalyzed by the

unique multicomponent enzyme system, methane monooxygenase (MMO). Two distinct types of MMO are known to exist in different cellular locations in methanotrophs, a cytoplasmic soluble form (sMMO) and a membrane-bound particulate form (pMMO). The latter has been found in all methanotrophs studied except the *Methylocella* species (Dedysh *et al*., 2000). Only some of the methanotrophs that have been tested possess both forms of MMO enzymes (Murrell *et al*., 2000a,b; Stainthorpe *et al*., 1990b). The occurrence of sMMO does not appear to be a taxonomic property of these bacteria since in five studied strains of haloalkalotolerant methanotroph *Methylomicrobium buryatense*, only in strain 5G, sMMO activity and *mmo*X (the gene encoding the active site of sMMO) were detected (Kalyuzhnaya *et al*., 2001). Among 11 strains of marine methanotrophs tested, only *Methylomicrobium* sp. NI had sMMO (Fuse *et al*., 1998). The limited diversity of sMMO-containing methanotrophs detected so far may be due to constraints on either genetic diversity of this enzyme or essential conservation of its function (Auman and Lidstrom, 2002; Heyer *et al*., 2002; McDonald *et al*., 2006). Remarkably, sMMO is not a constitutive enzyme and the only inducer for expression of sMMO is the ratio of Cu to biomass. Under a high Cu: biomass ratio (>2.5 μmol/g cell) the pMMO is synthesized, whereas at low ratios, the sMMO is synthesized (Stanley *et al*., 1983).

B. Soluble methane monooxygenase

sMMO catalyzes the NAD(P)H- and O_2-coupled conversion of methane to methanol in methanotrophic bacteria:

$$CH_4 + NAD(P)H + H^+ + O_2 \longrightarrow CH_3OH + NAD(P)^+ + H_2O$$

The best studied sMMO enzymes are from *Mc. capsulatus* Bath and *Methylosinus trichosporium* OB3b. They consist of three components: a hydroxylase, a reductase (MmoC), and a regulatory protein (MmoB) (Stirling and Dalton, 1979). The hydroxylase complex is a nonheme hexameric metalloprotein (250 kDa) in which α, β, and γ subunits have masses of 61, 45, and 20 kDa, respectively. The sMMO contains in the α subunit an unusual μ-hydroxobridged diiron center (Fox *et al*., 1989; Green and Dalton, 1985). Two Fe atoms linked via an oxygen bridge form the unique structure of this binuclear Fe-center, which not only acts as O-carrier but also has a catalytic function.

The reductase (MmoC) is a Fe-S flavoprotein (38.5 kDa) capable of carrying two electrons. During its reduction, intramolecular transfer of electrons from NAD(P)H to the Fe_2S_2 center of the reductase occurs. Then the electrons are transferred to the site of CH_4 oxidation and oxygen reduction on the hydroxylase complex. Thus, the reductase appears to

function as a one-electron transformase, providing a source of reductant from NADH.

The regulatory or effector protein (MmoB) is a small polypeptide (16 kDa) having neither metal nor prosthetic groups and is absolutely indispensable for the manifestation of hydroxylase activity in *Mc. capsulatus* Bath (Green and Dalton, 1985) and promotes this activity in *Ms. trichosporium* OB3b (Fox *et al.*, 1989). The MmoB fulfills a coupling of oxidation of NADH and CH_4, thus acting as a regulator of the activity triggering the enzyme from an NADH oxidase (in the absence of CH_4) to a hydroxylase (in the presence of CH_4).

The components of sMMO are organized into a stable protein complex, which is necessary for enzyme function (Fox *et al.*, 1989). The following mechanism for the reaction catalyzed by sMMO has been postulated as follows. Methane is binding with the active center of oxidized enzyme and appears to be solubilized in a hydrophobic pocket since a direct linkage for CH_4 and Fe^{3+} was not revealed. Then, one of the ferric atoms is reduced with formation of mixed valency (Fe^{2+}/Fe^{3+}) of the enzyme. The second one-electron reduction of the active center results in the production of active hydrogen peroxide (Fox *et al.*, 1989; Woodland and Cammack, 1985) followed by its cleavage in one of two possible reactions. Decomposition of H_2O_2 produces the hydroxyl radical $HO^{\bullet}$, which accepts a hydrogen from the methane molecule by Fenton reaction to form $CH_3^{\bullet}$ and H_2O, ultimately to produce methanol. Alternatively, heterolysis of H_2O_2 can yield H_2O and $O^{\bullet}$. The latter is able to accept hydrogen from CH_4, thus forming $CH_3^{\bullet}$, which produces methanol by recombination with $OH^{\bullet}$ through participation of Fe followed by regeneration of the oxidized form of binuclear Fe-center (Dalton, 2005; Fox *et al.*, 1989; Lieberman and Rosenzweig, 2004). Significant progress has been made during recent years in the study of sMMO catalysis through the discovery of reaction cycle intermediates (radicals) as well as by the use of spectroscopic techniques and diagnostic chemical reactions.

C. Particulate methane monooxygenase (pMMO)

pMMO is extremely unstable, which makes it difficult to work with biochemically. Only recently has pMMO of *Mc. capsulatus* Bath been purified and characterized (Kitmitto *et al.*, 2005; Nguyen *et al.*, 1994, 1998; Smith and Dalton, 1989). The enzyme is a trimer $(\alpha\beta\gamma)_3$ consisting of three subunits of approximately 45, 27, and 23 kDa. The active pMMO contains two Fe atoms and approximately 15 Cu atoms per enzyme molecule and all of them appear to participate in catalysis. Noteworthy, there is still some uncertainty as to the exact number of Cu present and also if Fe is present in the pMMO (reviewed by Lieberman and Rosenzweig, 2004). For the activity of the enzyme, a small fluorescent

chromopeptide methanobactin (mb) is required which is easily dissociated from the αβγ polypeptides of the MMO. This ''Cu-shuttle'' or Cu-mb appears to be involved in uptake and binding of different extracellular metals which are coordinated and reduced via a mechanism similar to that of copper (Choi *et al*., 2005, 2006; DiSpirito *et al*., 1998; Kim *et al*., 2004). Physiological reductants for pMMO are the cytochromes $b_{559/569}$ or c_{553}, whereas the artificial ones are duroquinol and NADH (Shiemke *et al*., 1995). Although sMMO has been well characterized, most questions regarding the molecular structure, chemical mechanism and regulation of pMMO remain unsolved (Dalton, 2005; Lieberman and Rosenzweig, 2004, 2005).

In the chromosome of *Mc. capsulatus* Bath, there are two copies of the pMMO gene cluster *pmo*C, *pmo*A, *pmo*B and an additional copy of *pmo*C (Semrau *et al*., 1995; Stolyar *et al*., 1999, 2001). In nitrifying bacteria, a similar duplication of the *amo*CAB genes, coding for ammonia monooxygenase (AMO), has also been established. A comparison of the *pmo* and *amo* genes sequences revealed a similar evolutionary linkage (Holmes *et al*., 1995; Klotz and Norton, 1998). A high degree of homology of pMMOs (80–94%) and duplication of *pmo*CAB genes were also shown in *Ms. trichosporium* OB3b and *Methylocystis* sp. N (Gilbert *et al*., 2000). Recently in type II methanotrophs two very different *pmoA* genes were found: conventional *pmoA* or *pmoA1* and novel *pmoA* or *pmoA*2 (Dunfield *et al*., 2002; Tchawa Yimga *et al*., 2003). In *Methylocystis* strain SC2, *pmoA1* and *pmoA2* gene copies are each part of a complete *pmoCAB* gene cluster (*pmoCAB1* and *pmoCAB2*), which exhibit low levels of identity at both the DNA level (67.4 to 70.9%) and the derived protein level (59.3 to 65.6%) but the secondary structures predicted for PmoCAB1 and PmoCAB2, as well as the derived transmembrane-spanning regions, are nearly identical (Ricke *et al*., 2004).

Transcription of the *pmo*CAB cluster is initiated from a start site located 300 bp upstream (5′) of *pmo*C, that is, from a σ^{70} factor, which may be downregulated during growth at a low concentration of Cu when there is enhanced expression of sMMO. In *Mc. capsulatus* Bath, six ORFs organized in one operon *mmo*XYBZDC, which encodes the structural genes for sMMO. The exact mechanism of reciprocal regulation of sMMO and pMMO synthesis by Cu ions is not known. Transcription of the *mmo* operon is initiated from a σ^{N}-(σ^{54})-dependent promoter, which requires an activating protein for creation of the transcriptional complex. In the *mmo* cluster of *Mc. capsulatus* Bath and *Ms. trichosporuim* OB3b, two new genes *mmo*R and *mmo*G were found. Noteworthy, MmoR, encoded by *mmo*R, is related to the class of regulatory proteins elevating the efficiency of linkage of the RNA polymerase σ^{N}-subunits (RpoN) with the promoter region, whereas MmoG is a homologue of the chaperone GroEL, and may be responsible for correct folding of the regulatory protein MmoR (Csaki *et al*., 2003; Gilbert *et al*., 2000; Stafford

et al., 2003). Recently two copies of *mmoX* have been observed in *Methylosinus sporium* 5; however, mutagenesis of the second copy of *mmoX*, which occurs on its own in the chromosome and is separate from the usual *mmoXYBZDC* cluster, showed that this second copy is not functional (Ali *et al*., 2006).

A similar model for transcriptional regulation implies positive control of expression of the sMMO genes. In the absence of copper, expression of *mmo*R and *mmo*G and correct folding of MmoR occurs. The latter forms a complex with σ^N subunits of RNA polymerase and facilitates transcription of *mmo*XYBZDC. Alternatively, at high copper levels, the MmoR is inactivated directly or via MmoG by an as yet unknown mechanism (possibly as a result of incorrect folding of the protein molecule) so that σ^N-dependent initiation of the *mmo* operon transcription does not occur. Interestingly, mutations of *rpo*N, *mmo*R, and *mmo*G genes have led to the inactivation of sMMO (Stafford *et al*., 2003). It has been suggested that synthesis of pMMO is regulated by a derepression mechanism in which at high copper levels the formation of the complex of hypothetic repressor molecule and a copper-binding regulatory protein might alleviate repression of pMMO expression from the *pmo*CAB gene cluster (Murrell *et al*., 2000a,b).

The sMMO catalyzes both oxidation of methane and transformation of a wide spectrum of aliphatic and aromatic hydrocarbons and their halogenated derivatives (Colby and Dalton, 1976; Fox *et al*., 1990; Grosse *et al*., 1999). Therefore, methanotrophs possessing sMMO play an important role in bioremediation of the polluted environments and still have considerable potential in biocatalysis and biotransformation (Dalton, 2005; Leak *et al*., 1985).

In contrast to sMMO, the pMMO oxidizes a restricted spectrum of carbon substrates (alkanes and alkenes up to C_5, Stirling *et al*., 1979); however when a methanotroph grows using pMMO, the efficiency of CH_4 conversion into biomass increased by 34% when compared with that of methanotrophs expressing only sMMO (Leak *et al*., 1985). Usually, pMMO is localized in the ICM and can comprise up to 80% of total membrane proteins (Nguyen *et al*., 1998). Copper is necessary for the expression and activity of pMMO enzyme. The pMMO generally has a high affinity for CH_4 (K_m = 1–2 μM) and O_2 (K_m = 0.1 μM). Often the sMMO is less active with CH_4 (K_m = 3 μM) and O_2 (K_m = 16.8 μM). NADH is required for methane oxidation by sMMO. Duroquinol serves as a reductant for pMMO *in vitro*. Finally, although sMMO and pMMO both oxygenate methane to methanol, they show no similarity in the amino acid sequences and requirements for metal cofactors. More details of the structure and function of these unique enzymes have been reviewed recently (Chan *et al*., 2004; Dalton, 2005; Hakemian and Rosenzweig, 2007; Lieberman and Rosenzweig, 2004).

The availability of X-ray crystal structures and development of a homologous system for the expression of highly active sMMO hydroxylase from *Ms. trichosporium* has made possible structure–function studies on the putative active site of sMMO. Key residues in the active site of sMMO including Cys151 and Thr213 and more recently Leu110 (a putative gating residue allowing access of substrate to the active site) have been mutated by site-directed mutagenesis (Smith *et al*., 2002; Borodina *et al*., 2007).

There is currently no expression system for the production of recombinant pMMO enzyme in an active form but some interesting work by Dalton and colleagues has recently been done on interactions with pMMO and methanol dehydrogenase (MDH). Using cryoelectron microscopy and single particle analysis to 16 Å resolution, Myronova *et al*. (2006) first reported the three-dimensional (3D) structure of a protein supercomplex that oxidizes methane to formaldehyde in *Mc. capsulatus* (Bath). These studies provided both the first insights into the supramolecular organization of a pMMO–MDH complexes and a deeper understanding of the structural basis for their function during bacterial growth under high copper concentrations. The authors proposed an original model in which the cylindric body of the trimeric enzymes tandem is formed by pMMO-H and the ''cap'' domain is formed by MDH. The postulated ''screw-cap'' mechanism for the assembly of two enzymes, pMMO and MDH, into a complex contributes to their stability and facilitates direct coupling of electron transport between them, thus leading to a more efficient utilization of CH_4 into biomass (Leak *et al*., 1985). Consistent with these results are the earlier observations of ICM supramolecular organization in methane-grown *Methylomonas methanica* and *Methylocystis echinoides* (Suzina *et al*., 1985). The membrane sheets were covered by hexagonally arrayed globular structures with lattice spacings of approximately 9.0 nm, while the free globular structures were 13 nm in diameter. Notably, these peculiar structures were absent in methanol-grown cells.

Overall, despite recent progress in characterization of pMMO, further research is required so as to understand the regulation, active site structure, and mechanism of this key enzyme in methanotrophs (Dalton, 2005; Hakemian and Rosenzweig, 2007; Lieberman and Rosenzweig, 2004).

D. Oxidation of methanol by methanol dehydrogenase

Methanol produced from the oxidation of methane is further oxidized by a periplasmic MDH, which has pyrroloquinoline quinone (PQQ) as a prosthetic group. The molecular structure and reaction mechanism of MDH have been well studied in gram-negative methanol utilizers (Anthony *et al*., 1994; Anthony and Williams, 2003; Van Spanning *et al*., 2000). MDH is a heterotetramer $\alpha_2\beta_2$ consisting of two large (67 kDa)

and two small (8.5 kDa) subunits. The enzyme has rather complicated stereostructure of radial symmetry, resembling an eight winged propeller composed from antiparallel segments of α-subunits. The PQQ molecule and Ca^{2+} ion are located in a funnel-like central channel. The β-subunit does not form a real domain in the MDH molecule and the function of this protein is still unclear (Anthony and Williams, 2003).

Oxidation of methanol is coupled with reduction of the prosthetic group (PQQ) into the corresponding quinol ($PQQH_2$) followed by two step transfer of electrons to the acceptor, which is an inducible cytochrome c_{551} (c_L), and further via the cytochromes c_{550} (c_n) and c_{552} to the terminal oxidase (Goodwin and Anthony, 1995).

The components of the methanol oxidation pathway are coded by at least 25 genes (Chistoserdova *et al*., 2003; Lidstrom, 1990). Lidstrom and colleagues have found that in the chromosome of *Methylobacterium extorquens* AM1, these genes are located in five gene clusters—*mxa*, *mxb, mxc, pqq*ABCDE, *pqq*FG—which, respectively, are connected with some additional genes in a sequence *mxa*FJGIRSACKLDEHB. Of these genes, *mxa*J, *mxa*R and *mxa*S, *mxa*D, *mxa*E and *mxa*H are required for the formation of active MDH, *mxa*G encodes cytochrome c_{551}, and the products of genes *mxa*ACKLD are involved in insertion of PQQ and Ca^{2+} ion into MDH. The polypeptide encoded by *mxa*B regulates transcription of the MDH genes. Two gene clusters, *pqq*ABCDE and *pqq*FG, are involved in the biosynthesis of PQQ. The product of *pqq*A is a peptide of 23–29 amino acids, which is the proposed precursor of PQQ, containing tyrosine and glutamate. Four genes, *mxb*DM and *mxa*QE, are involved in transcriptional regulation of the methanol oxidation system (Zhang and Lidstrom, 2003).

Remarkably, in the *Mc. capsulatus* Bath genome, three sets of genes encoding the structural homologues of MDH and proteins necessary for its catalytic function were found (Ward *et al*., 2004). Along with cluster *mxa*FJGIRSACKLDEHB coding for $\alpha_2\beta_2$ tetrameric structure of the MDH, a second MDH-like cluster *mxa*FJ is present but lacks the homologue *mxa*I, which usually encodes a small subunit of MDH. The function of *mxa*I is unknown and it is not clear whether *mxa*FJ cluster encodes the components active in methanol oxidation. The third cluster of genes required for MDH synthesis in *Methylobacterium extorquens* AM1, consists of *mxa*ACKL (Zhang and Lidstrom, 2003).

The MDH enzymes purified and crystallized from *Methylomonas methanica* and *Methylosinus sporium* were monomeric (Mr = 60 kDa), thus indicating a possible presence of isozymes or some structure–function differences between MDH of methanotrophs and methylotrophs (Patel and Felix, 1978, Patel *et al.,* 1978). The recent finding of the multiple isozymes of MDH in *Methylophaga* spp. (Chang *et al*., 2002) calls for further comparative studies to explore more on this topic.

The genes coding for large subunits of MDH (*mxa*F) are highly conserved in methanotrophs and methylotrophs (Bastien *et al*., 1989; McDonald and Murrell, 1997). Oxidation of methanol is accompanied by the generation of a proton gradient (ΔpH) since the released protons are retained in the periplasm and the electrons are transferred by the cytochrome components through a cytoplasmic membrane to the terminal oxidase aa_3, where electrons are accepted by oxygen with concominant consumption of protons from the cytoplasm (Chetina and Trotsenko, 1986; Sokolov *et al*., 1981). The efficiency of electron transfer from MDH to oxygen ($\rightarrow H/O = 3.0–3.5$) implies the operation of several proton translocating segments (Tonge *et al*., 1975, 1977; Trotsenko and Chetina, 1988).

E. Oxidation of formaldehyde by a linear pathway

Formaldehyde is the key intermediate in the linear pathway of oxidation of methane to CO_2. It is also cytotoxic. A major portion of the reducing equivalents required for methane oxygenation is formed during formaldehyde oxidation via formate to CO_2. Methanotrophs have several enzymes involved in formaldehyde oxidation. These enzymes are divided into two groups: the $NADP^+$-specific and the cytochrome-linked enzymes. In turn, the $NADP^+$-specific dehydrogenases are divided into three subgroups depending on the secondary cofactors used, such as thiols (GSH), tetrahydrofolates (THF, H_4F), or H_4MPT.

In *Mc. capsulatus* Bath grown at high copper levels (>2.5 μM Cu per mg of cell protein), that is, under conditions of pMMO expression, formaldehyde is mainly oxidized by a particulate cytochrome-linked formaldehyde dehydrogenase (FADH) (Zahn *et al*., 2001). The enzyme specific to formaldehyde is a homotetramer (Mr = 49.5 kDa) having PQQ as prosthetic group, and uses coenzyme Q_8 or the complex of cytochromes $b_{559/569}$ as the physiological acceptors of electrons. FADH-linked cytochromes that *in vitro* use dyes as the artifical electron acceptors were found in many methanotrophs and methylotrophs. However, it has long been considered that these FADH enzymes may not be physiologically significant since their activity is usually not high enough. As shown recently, in *Mc. capsulatus* Bath, the activity of FADH is determined by the assay conditions used. In the presence of the complex of cytochromes $b_{559/569}$, the electrons preferably reduce the cytochrome complex, but not 2,4-dichlorophenol indophenol, which is usually used for the enzyme assay *in vitro*. Also, the enzyme activity was inhibited in the presence of Tris-HCl (Zahn *et al*., 2001). The electrons enter the respiratory chain at the level of cytochrome *b*/ubiquinone. Hence, the coupling between the respiratory chain and pMMO might occur at the level of the cytochrome complex bc_1, possibly via coenzyme Q_8 (Myronova *et al*., 2006).

F. Pterin-dependent oxidation of formaldehyde

As found recently, methylotrophs use pterin cofactors, H_4F/THF and H_4MPT, for activation of formaldehyde oxidation (Vorholt, 2002). The H_4F-dependent pathway of CH_2O oxidation includes the following reactions:

$$CH_2O \overset{H_4F}{\longleftrightarrow} N^5, N^{10} - CH_2 = H_4F \overset{MtdA}{\underset{NADP^+}{\longleftrightarrow}}$$

$$N^5, N^{10} - CH \equiv H_4F \overset{Fch}{\longleftrightarrow} N^5 - CHO - H_4F \overset{Fhs}{\longleftrightarrow} HCOOH \overset{FDH}{\longrightarrow} CO_2$$

In type II methanotrophs, the major role of the THF-pathway enzymes is to maintain the high concentration of N^5,N^{10}-methylene-THF, which is the primary acceptor of formaldehyde in the serine pathway of C_1 assimilation. Because of the reversibility of the reactions catalyzed, these enzymes can be regulated by the requirements of the cell either into the oxidation or assimilation of formaldehyde (Vorholt, 2002). The functions of the THF pathway enzymes have not been elucidated in type I methanotrophs. Presumably, the conversion of C_1 units as THF derivatives occurs in almost all organisms and C_1 units are used primarily in biosynthesis of thymidine and purines.

The reactions of H_4MPT-dependent oxidation of formaldehyde were first found in methanogenic and sulfate reducing Archaea and were considered to be characteristic of these strict anaerobes. The recent discovery of the H_4MPT-dependent oxidation of formaldehyde in the aerobic facultative methylotroph *Methylobacterium extorquens* AM1 (Chistoserdova *et al*., 1998) has opened up exciting new possibilities for the evolutionary relationships between aerobes and anaerobes, that is, Eubacteria and Archaea. The H_4MPT-dependent pathway of formaldehyde oxidation includes the following reactions:

$$CH_2O \underset{H_4MPT}{\overset{Fae}{\longleftrightarrow}} N^5, N^{10} - CH_2 = H_4MPT \overset{MtdA/B}{\underset{NAD(P)^+}{\longleftrightarrow}}$$

$$N^5, N^{10} - CH \equiv H_4MPT \overset{Mch}{\longleftrightarrow} N^5 - CHO - H_4MPT \underset{H_4MPT}{\overset{Ftr}{\longleftrightarrow}} HCOOH \underset{NAD^+}{\overset{FDH}{\longrightarrow}} CO_2$$

The highest activities of the H_4MPT pathway enzymes, methenyl-H_4MPT cyclohydrolase and NAD(P)-dependent methenyl-H_4MPT dehydrogenase, are found in *Mc. capsulatus* Bath and other α- and γ-methanotrophs (Vorholt *et al*., 1999). Although detailed studies have been done only for *Methylobacterium extorquens* AM1, the genes encoding these enzymes occur in one cluster and have sequences with a high degree of identity to those of methanogens. Hence, four enzymes are involved in oxidation of formaldehyde to formate through the H_4MPT pathway.

Formaldehyde activating enzyme (Fae), a novel homotrimeric enzyme (Mr = 18 kDa), in *M. extorquens* and *Paracoccus denitrificans*, accelerates the

spontaneous reaction of condensation of formaldehyde and H_4MPT with the formation of N^5,N^{10}-methylene-H_4MPT (Vorholt *et al*., 2000). Mutant Fae^- was more sensitive to formaldehyde and unable to grow on methanol, thus indicating an important role for this enzyme. So far, the methanotrophs have not been tested for its activity. However, the existence of two Fae homologues from *Methylosinus* sp. LW2 (83% identity) might be due to a lateral gene transfer or recent gene duplication events since they are adjacent on the chromosome (Chistoserdova *et al*., 2004).

Oxidation of methylene-H_4MPT to methenyl-H_4MPT is catalyzed by two different enzymes. An $NAD(P)^+$-specific enzyme (MtdA) also catalyzed the reversible dehydrogenation of methylene-THF (Vorholt *et al*., 1998). However, the $NAD(P)^+$-dependent methylene-H_4MPT dehydrogenase (MtdB), which is specific to methylene-H_4MPT but nonactive with methylene-THF, catalyzes an irreversible reaction (Hagemeier *et al*., 2000). This is the first member of the new family of THF/H_4MPT dehydrogenases which are different from the F_{420}-dependent methylene-H_4MPT dehydrogenase of methanogenic and sulfate reducing Archaea. The inability to obtain any viable $MtdA^-$ mutants (Hagemeier *et al*., 2000) clearly points to the involvement of this enzyme in central C_1 metabolism. At the same time, the $MtdB^-$ mutants did not grow on methanol and were very sensitive to CH_2O but grew well on succinate. It was suggested that MtdB converts N^5,N^{10}-methylene-H_4MPT *in vivo*, whereas MtdA operates mainly as a THF-dependent enzyme. This was confirmed by the location of these genes: *mtd*A is located near *fch*A encoding the THF-dependent cyclohydrolase while *mtd*B is found in the same cluster of genes encoding the other H_4MPT-dependent enzymes. Very recently, a novel class of methylene-H_4MPT dehydrogenase (MtdC) was found (Vorholt *et al*., 2005).

Methenyl-H_4MPT-cyclohydrolase (Mch) catalyzes conversion of methenyl-H_4MPT into N^{10}-formyl-H_4MPT. The enzyme from *Methylobacterium extorquens* AM1 is monofunctional and has no chromophoric prosthetic group. It is activated (20×) in the presence of 1.2 M KCl which is not a characteristic of Mch from Proteobacteria, but more peculiar to Mch of Archaea (Vorholt, 2002).

Formyltransferase/hydrolase complex (FhcABCD) of *Methylobacterium extorquens* AM1 contains four different components and catalyzes conversion of N^{10}-formyl-H_4MPT to formate. One of the components (FhcD) corresponds to formyl transferase of Archaea (with sequence identity of about 40%) and catalyzes transfer of the formyl group from N^{10}-formyl-H_4MPT to methanofuran (Pomper and Vorholt, 2001). The analogue of methanofuran was not found in aerobic methylotrophs but was shown to be active with methanofuran from Archaea. Other subunits of the Fhc complex have sequences similar to those of formyl-methanofuran dehydrogenase from Archaea (FmdA, FmdB, and FmdC) but were not related to the family of molybdenum-dependent dehydrogenases of

Archaea. The FhcABCD complex of *Methylobacterium extorquens* AM1 hydrolyzes formyl-methanofuran to formate and methanofuran. The active center of hydrolysis is localized in the Fch subunit. The genes encoding FhcABCD are located nearby and are required for bacterial growth on methanol (Chistoserdova *et al*., 1998). Oxidation of formaldehyde to formate in the H_4MPT pathway, because of the irreversibility of the reaction, is an effective catabolic process generating NAD(P)H. However, its role must be clarified, especially because of the presence of $NAD(P)^+$-independent formaldehyde dehydrogenase at least in some methanotrophs.

For many years, cell extracts and purified enzyme preparations of *Mc. capsulatus* Bath were considered to contain a soluble $NAD(P)^+$-dependent formaldehyde dehydrogenase, the substrate specificity and kinetics of which might be regulated by a thermostable protein termed modifin (8.6 kDa) (Stirling and Dalton, 1978; Tate and Dalton, 1999). More recently, this enzymic preparation has been classified as a mixture of a quinoprotein MDH and $NAD(P)^+$-dependent methylene-H_4MPT dehydrogenase. Nevertheless, the existence of a soluble $NADP^+$-dependent formaldehyde dehydrogenase in this organism cannot currently be excluded (Adeosun *et al*., 2004).

G. Oxidation of formate to CO_2

This reaction is a final step in the methane oxidation pathway. In all extant methanotrophs, an NAD^+-dependent formate dehydrogenase (FDH) is present (Chetina and Trotsenko, 1981; Popov and Lamzin, 1994). The enzyme from *Ms. trichosporium* OB3b functions *in vitro* as an electron donor for sMMO (Yoch *et al*., 1990) or nitrogenase (with the additional participation of ferredoxin-NAD^+ reductase and ferredoxin) (Chen and Yoch, 1988, 1989). In this methanotroph, NAD^+-FDH consists of two types of polypeptides (54 and 106 kDa), which can be combined to form di- and tetramers ($\alpha\beta$ and $\alpha_2\beta_2$), of which, the tetramer (Mr = 315 kDa) seems to be the most dominant one. The enzyme contains nonheme iron (11–18 g-atom/mol protein) and acid labile SH-groups (15–20 mol/mol protein). FDH was most active at a pH of 6.5–7.5 and had low K_m values for formate (146 μM) and NAD^+ (200 μM) but was stabilized and activated by the addition of FMN (K_m = 0.02 μM). In cell extracts of many methanotrophs, a highly active phenazine methosulfate-linked FDH associated with membranes was found. Perhaps this activity belongs to the formaldehyde oxidizing enzyme that has a different substrate specificity (Chetina and Trotsenko, 1981). It should be noted that in the genome of *Mc. capsulatus* Bath there were ORFs encoding FDH-like proteins and in the future, their role must be elucidated (Ward *et al*., 2004). For example, three different FDHs are functional in *M. extorquens* AM1 and their

expression is regulated at least in part by the presence of molybdenum or tungsten (Chistoserdova *et al*., 2004).

IV. PATHWAYS OF PRIMARY C_1 ASSIMILATION AND INTERMEDIARY METABOLISM

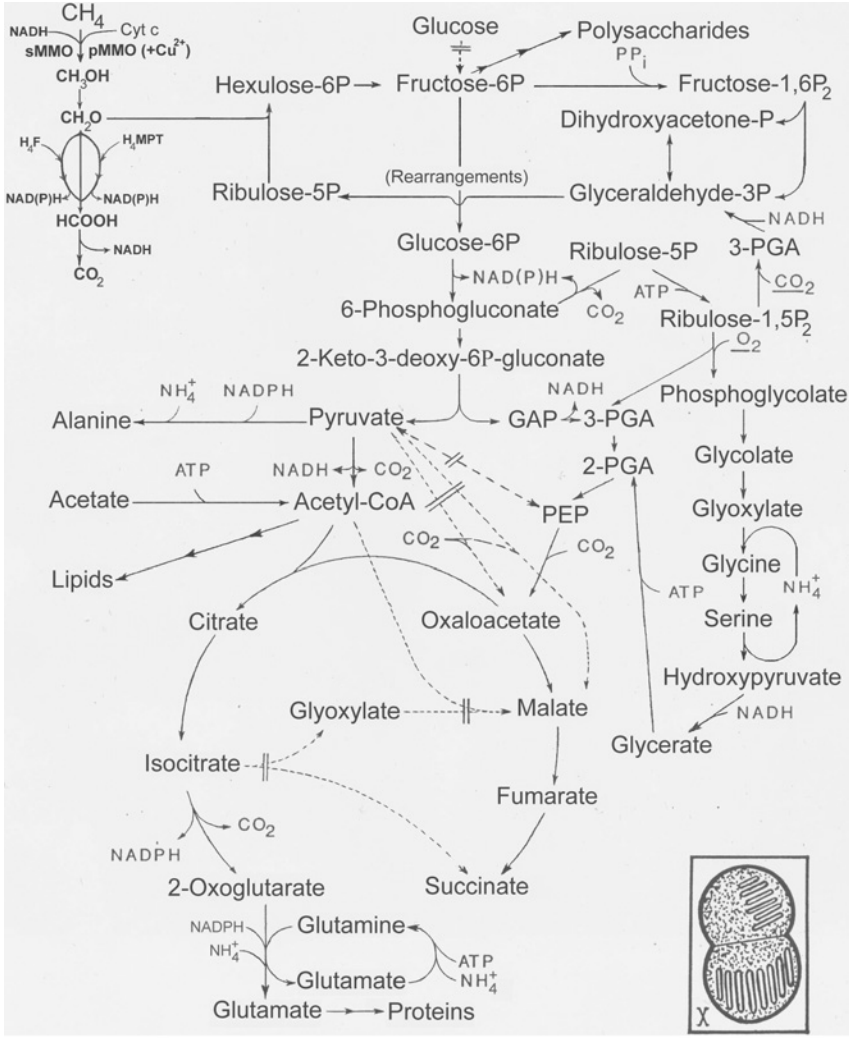
A. Assimilation of formaldehyde via the Quayle ribulosemonophosphate and serine pathways

Thanks to the classical radioisotopic and enzymatic studies by Quayle and colleagues, it is known that methanotrophs employ two major pathways of primary C_1 assimilation: the RuMP and serine cycles (Quayle, 1969, 1972, 1980). In both these cycles, phosphotrioses are synthesized from formaldehyde, which is the key intermediate in carbon metabolism (Fig. 5.1).

In the first part of the RuMP cycle, formaldehyde is fixed with ribulose-5-phosphate to form (D-arabino)-3-hexulose-6-phosphate in a reaction catalyzed by 3-hexulosephosphate synthase (HPS). This very unstable product is rapidly isomerized to fructose-6-phosphate by phosphohexuloisomerase (PHI) (Ferenci *et al*., 1974). These two specific enzymes catalyze the formation of both C–C bonds and phosphohexoses in type I and type X methanotrophs. In thermotolerant *Mc. capsulatus* Bath, the purified HPS is a homohexameric (310 kDa) membrane-bound enzyme with an unusually large subunit size (49 kDa). On the basis of the genome sequence, the HPS molecular mass appears to correspond to the product of the *hps-phi*-fused gene that may be generated by a tandem duplication (Ward *et al*., 2004). In general, HPS is a member of the orotidine 5′-monophosphate decarboxylate suprafamily (30% identity) and like other members of this suprafamily, requires Mg^{2+} or Mn^{2+} for activity. Although the structure of HPS has not yet been solved, based on its primary amino acid sequence, it is expected to share the $(\beta/\alpha)_8$-barrel fold (reviewed by Kato *et al*., 2006).

By comparison with HPS, the characteristics of PHI are poorly documented. The highly active purified enzyme was shown to exist as a homodimer (~40 kDa) and is sensitive to inhibition by many bivalent metal ions, Cu^{2+} in particular. The HPI protein crystallized from the methanogen *Methanocaldococcus jannaschii* has an α/β structure consisting of a five-stranded parallel β-sheet flanked on both sides by α helices, forming a three-layered α-β sandwich (reviewed by Kato *et al*., 2006).

Conversely, in methylotrophs, HPS is a soluble mono- or dimeric enzyme (32–47 kDa) (Sokolov and Trotsenko, 1979). Recently, the genes encoding these enzymes (*hps* and *hpi*) have been cloned and sequenced in obligate methylotroph *Methylobacillus aminofaciens* 77a, facultative



Pathways of primary and intermediary metabolism in Type X obligate methanotrophs

FIGURE 5.1 Pathways of primary and intermediary metabolism in type X obligate methanotrophs.

Mycobacterium gastri MB19 and thermotolerant *Bacillus brevis* S1 (Kato *et al*., 2006; Mitsui *et al*., 2000; Sakai *et al*., 1999). In most cases, organisms have both HPS and HPI orthologues, implying that these two genes may have evolved as a pair to participate in detoxification and assimilation of formaldehyde via the RuMP pathway.

Recent genomic and biochemical studies have revealed a novel metabolic function of HPS and HPI, which are involved in pentose phosphate biosynthesis in some archaeal strains lacking genes for NAD(P)-dependent glucose-6-phosphate and 6-phosphogluconate dehydrogenases, transaldolase, transketolase, and ribulose-5-phosphate epimerase. Hence, this enzyme system plays different physiological roles in Bacteria and Archaea, thus catalyzing the forward and reverse reactions, respectively (reviewed by Kato *et al*., 2006).

In the second part of the RuMP cycle, the phosphohexoses are split to (phospho)trioses. Only in methanotrophs this cleavage occurs by two simultaneous pathways, via the Entner–Doudoroff and Embden–Meyerhof–Parnas variants. In the first variant, fructose-6-phosphate is converted via glucose-6-phosphate and 6-phosphogluconate into 2-keto-3-deoxy-6-phosphogluconate (KDPG), which is subsequently cleaved by KDPG-aldolase to pyruvate and glyceraldehyde-3-phosphate (GAP). Alternatively, in the second variant fructose-6-phosphate is phosphorylated by ATP into fructose-1,6-bisphosphate, followed by aldolase cleavage to GAP and dihydroxyacetone-phosphate, the latter being isomerized to GAP. Nontheless, the glycolytic cleavage of phosphohexoses into phosphotrioses has not been considered as a physiologically significant pathway in methanotrophs because of the low or zero activity of ATP-dependent 6-phosphofructokinase (Strom *et al*., 1974). However, the discovery of very active pyrophosphate-dependent 6-phosphofructokinase (PP_i-PFK), together with the high intracellular levels of PP_i (up to 20 mM), has established that PP_i, but not ATP, is the phosphoryl donor in this reaction (Trotsenko and Shishkina, 1990; Trotsenko *et al*., 1996). Further, the purified PFK of *Methylomonas methanica* 12, being a homodimer (2×45 kDa), is rather similar in kinetic and regulatory properties to the analogous enzymes from microbial rather than plant origin (Beschastny *et al*., 1992).

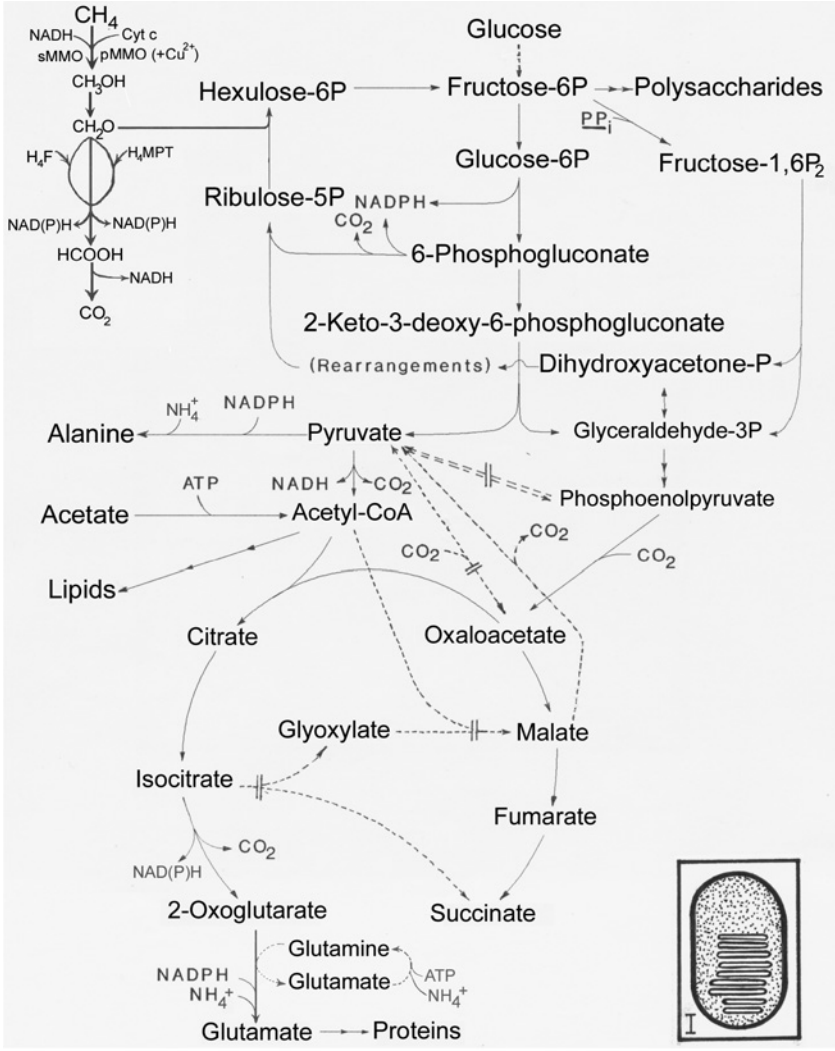
The recent identification and cloning of the *pfk* gene revealed its distant similarity (16.5% of identical amino sequences) to that of *Methylococcus capsulatus* Bath and the facultatively methylotrophic actinomycete *Amycolatopsis methanolica* (23.2%) (Reshetnikov *et al*., 2005). This finding suggests a different origin and metabolic role for the PP_i-PFKs in these bacteria (Alves *et al*., 2001). The high degree of divergence of the *pfk* gene in methanotrophs may be determined by the characteristics of primary and central metabolism in these bacteria, as well as with differences in the function and activity of the PP_i-PFK. Significantly, PP_i-PFK, having a crucial position at metabolic cross-roads, catalyzes easily reversible interconversion of fructose-6-phosphate into fructose-1,6-bisphosphate and participates in distribution of the carbon flux between the glycolytic and KDPG segments (branches) of the RuMP cycle. In fact, this metabolic loop serves to balance the levels of GAP and (phosphoenol)pyruvate in the cell (Trotsenko *et al*., 1996).

In the third part of the RuMP cycle, the primary acceptor of formaldehyde, that is, ribulose-5P is regenerated from glyceraldehyde-3P and fructose-6P in a series of transaldolase/transketolase reactions analogous to photo- and chemotrophic bacteria. It has been postulated, but not experimentally proven, that the RuMP pathway is an evolutionary predecessor/ancestor of the Calvin–Benson–Basham (CBB) cycle (Lawrence *et al*., 1970; Quayle and Ferenci, 1978).

Type II methanotrophs employ the serine cycle for C_1-assimilation (Fig. 5.2). In the first part of the serine cycle, formaldehyde (after condensation with THF and formation of N^5,N^{10}-methylene THF) reacts with glycine to produce serine by the action of the appropriate serine-hydroxytransmethylase (SHTM). The amino group of serine is then transferred by a specific serine-glyoxylate aminotransferase (SGAT) to glyoxylate, thus forming glycine and hydroxypyruvate (by hydroxypyruvate reductase, HPR), which is phosphorylated by ATP-glycerate kinase to 2-phosphoglycerate, followed by isomerization to PEP and its subsequent carboxylation to oxaloacetate. The reduction of oxaloacetate, catalyzed by malate dehydrogenase, forms malate which is then converted to malyl-CoA by malate thiokinase. Finally, malyl-CoA lyase forms glyoxylate and acetyl-CoA, the latter being a primary product of the serine cycle. Consequently, SHTM, SGAT, HPR, and malyl-CoA lyase are the key indicative enzymes of the serine cycle.

In the second part of the serine cycle, acetyl-CoA is oxidized into glyoxylate which is further (trans)aminated to glycine, so that the primary acceptor of formaldehyde is regenerated. Interestingly, the obligate methanotrophs and serine pathway methylotrophs ''lacked'' isocitrate lyase (icl^- variant). As shown recently, glyoxylate can be regenerated via the formation of acetoacetyl-CoA and hydroxybutyryl-CoA, the known intermediates of the poly-β-hydroxybutyrate biosynthesis pathway, and also crotonyl-CoA and butyryl-CoA, the intermediates of fatty acid biosynthesis (Korotkova and Lidstrom, 2002). However, at present it is not clear whether this rather complicated pathway proposed for *M. extorquens* AM1 or other variants (via ethylmalonyl-CoA citramalate or methylmalate) operate in type II methanotrophs (Erb *et al*., 2007; Meister *et al*., 2005).

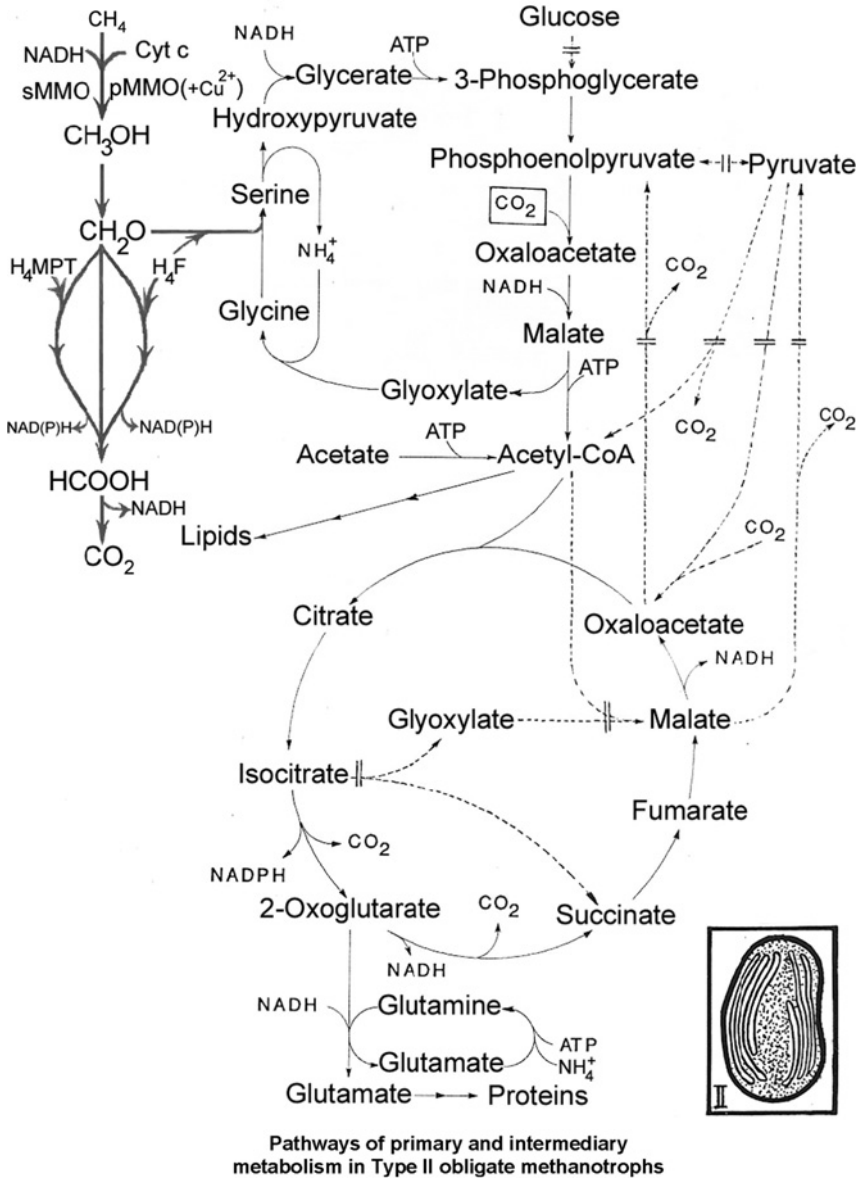
The type X methanotrophs (Fig. 5.3), belonging to the genera *Methylococcus* and *Methylocaldum,* assimilate formaldehyde mainly by the RuMP pathway although they also possess less active enzymes for the serine pathway and the CBB-cycle, that is, phosphoribulokinase and ribulosebisphosphate carboxylase/oxygenase (Rubisco) (Stanley and Dalton, 1982; Taylor, 1977; Taylor *et al*., 1981). The role of these enzyme activities is still unclear and their increase at elevated growth temperatures suggests that the supplementary energy consuming pathways operate to dissipate excess metabolic energy in order to maintain the energetic status of the cell and protect against overheating (Eshinimaev *et al*., 2004).



Pathways of primary and intermediary metabolism in type I obligate methanotrophs

FIGURE 5.2 Pathways of primary and intermediary metabolism in type I obligate methanotrophs.

As shown, in type I methanotrophs, 5–15% of the carbon in cell biomass is derived from CO_2 while in type II methanotrophs it is up to 50%. Moreover, in both type I and type II methanotrophs, PEP carboxylase is responsible for anaplerotic CO_2 fixation (Shishkina and Trotsenko, 1986). In type X methanotrophs (*Methylococcus* and *Methylocaldum*), the



Pathways of primary and intermediary metabolism in Type II obligate methanotrophs

FIGURE 5.3 Pathways of primary and intermediary metabolism in type II obligate methanotrophs.

CBB cycle enzymes are also involved in CO_2 fixation. Taylor *et al*. (1980) estimated that Rubisco of *Mc. capsulatus* Bath had an $\alpha_6\beta_6$ structure which differed from the typical hexadecameric structure ($\alpha_8\beta_8$) of the form I

Rubisco formed in Proteobacteria and also in cyanobacteria and higher plants. The Rubisco genes encoding the large subunit (*cbb*L), small subunit (*cbbS*), and putative regulatory gene (*cbbQ*) were shown to be located on one cluster in *Methylococcus* and *Methylocaldum* (Baxter *et al*., 2002). These facts confirm earlier postulated linkage of primary pathways of C_1 metabolism in methanotrophs and autotrophs (Quayle and Ferenci, 1978; Whittenbury, 1980).

B. Pathways of nitrogen assimilation

For a long time the ability to fix dinitrogen (N_2) was considered to be characteristic of only type II and type X methanotrophs (Hanson and Hanson, 1996; Murrell and Dalton, 1983a; Toukdarian and Lidstrom, 1984). However, recent research using PCR and acetylene reduction method has revealed the potential for N_2 fixing ability among other methanotrophs. The *nif* H genes coding for Fe-containing protein of nitrogenase and enzyme activity was also found in some type I methanotrophs such as *Methylomonas* and *Methylobacter*. Furthermore, phylogenetic analysis of amino acid sequences encoding by *nif* H genes has corroborated some differences correlating with separation of methanotrophs into two major types (Auman *et al*., 2001; Bulygina *et al*., 2002; Dedysh *et al*., 2004; Oakley and Murrell, 1988). The structural genes for nitrogenase of *Mc. capsulatus* Bath (*nifH, nifD*, and *nifK*) were previously shown to be contiguous as they are in other diazotrophs (Oakley and Murrell, 1991). Genome analysis extended this contiguous region to include other *nif* genes *nifN* and *nifX*, which are involved in synthesis of the nitrogenase iron-molybdenum cofactor. Two 2Fe-2S ferredoxins and two genes identified as conserved hypotheticals are interspersed with the *nif* genes in the same orientation. Similar organization of nitrogenase operons has been found in some other nitrogen-fixing Bacteria and Archaea (Ward *et al*., 2004).

Earlier, a correlation between carbon and ammonia assimilatory pathways has been found (Shishkina and Trotsenko, 1979). Type I methanotrophs assimilated NH_4 mainly by reductive amination of pyruvate or α-ketoglutarate, whereas type II methanotrophs used the glutamate cycle, that is, glutamine synthetase (GS) and the glutamine-oxoglutarate amidotransferase (GOGAT) system. Interestingly, in *Mc. capsulatus* Bath and type I methanotrophs grown on medium containing ammonia, the reductive amination of pyruvate (via alanine dehydrogenase) or α-ketoglutarate (via glutamate dehydrogenase) was prevalent under high ammonia growth conditions. In contrast, when grown under N_2-fixing conditions, that is, under ammonium limitation ($<$0.5 mM) or on medium containing nitrate (in the absence of ammonium) these methanotrophs assimilated ammonia via the glutamate cycle (Murrell and Dalton, 1983b). Four predicted ammonium transporters were identified in the

Mc. capsulatus Bath genome, confirming that ammonium is an important nitrogen source (Ward *et al*., 2004).

The GS purified from *Mc. capsulatus* Bath is a homododecameric enzyme (617 kDa) with a subunit molecular mass of 50 kDa (Murrell and Dalton, 1983a). As in heterotrophic bacteria, GS activity is regulated by (de) adenylylated mechanisms. At concentrations of >0.5 mM NH_4 in the medium, GS exists in the non-active adenylylated form. The structural gene for GS from *Mc. capsulatus* Bath has been cloned and sequenced. Its nucleotide sequence has 59% similarity with *gln*A gene of *Anabaena* sp. 7120. Regulation of *gln*A in this methanotrophs is analogous to that of enterobacteria and occurs via the Ntr system (Cardy and Murrell, 1990).

The levels of GS/GOGAT, glutamate, and alanine dehydrogenases in methanotrophs are also dependent on the nitrogen content in growth medium. The GOGAT of type I methanotrophs is specific to NADPH and is rather unstable, whereas in type II methanotrophs this enzyme is more stable and specific for the cofactor NADH (Murrell and Dalton, 1983c).

C. Biochemical basis/rationale of obligate methanotrophy

Obligate methanotrophs are unable to use polycarbon (non-C_1) compounds as growth substrates. By analogy with obligate autotrophy, there are several hypotheses to explain such a phenomenon (Shishkina and Trotsenko, 1982; Smith and Hoare, 1977; Whittenbury and Kelly, 1977; Wood *et al*., 2004). Previously, the dependence of methanotrophs on C_1 compounds was connected with pecularities of their cell wall and cytoplasmic membrane, that is, the absence of the appropriate transport systems for multicarbon (C_n) organic compounds. However, a stimulatory or inhibitory effect of some organic compounds on methanotrophic growth clearly demonstrated their transport through the cell wall and cytoplasmic membrane, followed by their involvement in the metabolism of both Type I and Type II methanotrophs (Eccleston and Kelly, 1972; Patel, 1975, 1977; Wadzinski and Ribbons, 1975; Xing *et al*., 2006).

The inhibitory effect of some amino acids on the growth of obligate methanotrophs also influenced a specific regulation of their metabolism since this effect was removed in the presence of other amino acids (Eccleston and Kelly, 1973a,b; Pinchuk *et al*., 1987). Unlike *E.coli*, the obligate methanotrophs have no isozymes of aspartokinase sensitive to each amino acid of the aspartate family (Malashenko *et al*., 1987). Perhaps, in obligate methanotrophs there is polyvalent repression by lysine and threonine of the activity of the first steps of the branched biosynthetic pathways for the aspartate family amino acids. However, similar regulation of the pathways of carbon and nitrogen metabolism may be a consequence, rather than the cause, of the obligate nature of methanotrophs for C1 compounds.

As shown in Table 5.2, the enzyme profiles of obligate methanotrophs support one attractive hypothesis explaining their inability to grow on polycarbon substrates because of the occurrence of multiple enzymic lesions, that is, the simultaneous absence of the activity of several key enzymes involved in central metabolism that are required to generate energy and reducing equivalents for growth and biosynthesis (Shishkina and Trotsenko, 1982).

In type I methanotrophs, functionally active α-ketoglutarate dehydrogenase as well as isocitrate lyase and malate synthase are absent, so oxidation of acetyl-CoA to CO_2 via the incomplete tricarboxylic acid (TCA) cycle and a deficient glyoxylate shunt is impossible (see Fig. 5.2). Such a ''horse shoe-like'' TCA cycle consists of two enzymatic sequences having primarily anabolic functions. In the TCA oxidative part of the ''horse shoe,'' CO_2 and NAD(P)H, α-ketoglutarate, and the glutamate family amino acids are formed whereas its reducing part gives oxaloacetate, succinate, and then porphyrins. These peculiarities of the incomplete TCA cycle and nonfunctional glyoxylate bypass were consistent with the results of earlier radioisotopic experiments when ^{14}C-acetate was incorporated mainly into lipids and glutamate family of amino acids (Patel *et al*., 1975, 1977; Wadzinski and Ribbons, 1975; Whittenbury and Kelly, 1977).

The recent genomic data are consistent with an oligotrophic lifestyle for *Mc. capsulatus* Bath, revealing a relatively limited array of membrane transporters for organic carbon compounds, for example, one complete and one partial ATP-binding cassette (ABC) family with predicted specificity for sugar uptake were identified along with components of transporters for peptides, carbohydrates and some organic and amino acids (Ward *et al*., 2004).

In type II methanotrophs with the serine pathway, α-ketoglutarate dehydrogenase is present, that is, there is a complete set of the TCA cycle enzymes (Table 5.2). These bacteria also have acetyl-CoA synthetase providing an initial involvement of acetate in the TCA cycle, so that, in principle, they can obtain metabolic energy and reducing equivalents from acetate. However, due to the lack of the glyoxylate bypass enzymes—isocitrate lyase and malate synthase—the formation of C_3- and C_4-intermediates from C_2-compounds is not possible. In addition, in type II methanotrophs, there is no activity of the E_1 component of pyruvate dehydrogenase complex as well as pyruvate kinase, pyruvate carboxylase, phosphoenolpyruvate (PEP) synthetase, and pyruvate phosphate dikinase, which leads to a limited metabolism of pyruvate (Fig. 5.3). Remarkably, the gluconeogenic pathway from C_3- and C_4-compounds (by means of pyruvate carboxylation to malate followed by its decarboxylation to PEP) is also inoperative because of the lack of pyruvate carboxylase and PEP carboxykinase in type II methanotrophs. Moreover, the low

activity of α-ketoglutarate dehydrogenase suggests an anabolic role for the TCA cycle in these bacteria (Shishkina and Trotsenko, 1982).

Because of the long-term difficulties in generating mutants of obligate methanotrophs, the aforementioned hypotheses on the causes of C_1-obligateness are still debatable. The appropriate evidence for the key role of pyruvate and α-ketoglutarate dehydrogenases in the catabolism of many organic compounds containing C–C bonds have been provided by the experiments with restricted facultative methylotrophs *M. extorquens* AM1 and *Hyphomicrobium* sp. X. Notably, their *kdh*$^-$ and *pdh*$^-$ mutants did not grow on multicarbon compounds including acetate (Bolbot and Anthony, 1980; Dijkhuizen *et al*., 1984; Taylor and Anthony, 1976).

A distinctive feature of methanotrophic metabolism is the generation of energy from methane for growth. Oxidation of CH_4 appears to require a specific regulation of electron flow from NADH to O_2 (Tonge *et al*., 1975). Since obligate methanotrophs have rather low or zero activity of NADH oxidase (the enzyme system transferring electrons from NADH to O_2) an alternative explanation of C_1-obligateness has been suggested. The unusual mechanisms of energy generation from C_1-compounds were postulated to be connected with the operation of a nontypical electron transfer chain in obligate methanotrophs. The electrons formed during methanol oxidation by periplasmic MDH are accepted by the cytochromes *c*-type and transferred through a terminal oxidase aa_3 to oxygen (Sokolov *et al*., 1981). The protons released are retained in the periplasm, whereas the electrons are transported through the cytoplasmic membrane by cytochrome components to the terminal oxidase and accepted by O_2 with the comcomitant consumption of cytoplasmic protons (Sokolov *et al*., 1981). This is accompanied by the formation of a proton gradient (ΔpH) across the cytoplasmic membrane and thus provides energy for the growth and activity of methanotrophs (synthesis of ATP, transport of substrates, and cell motility). The required reducing equivalents are generated in the subsequent reactions of formaldehyde oxidation by formaldehyde and formate dehydrogenases as well as the H_4MPT pathway enzymes. Consequently, the metabolism of obligate methanotrophs (and methylotrophs) is focused on the generation of energy and reducing equivalents for biosynthesis from C_1 substrates. During evolution, this might lead to a loss of specific mechanisms of the energy transformation, transport of polycarbon compounds and regulation as well as to a repression or loss of the genes encoding some enzymes of central metabolism (Sokolov and Romanovskaya, 1992).

Answers to the question about the true causes of C_1 obligateness in methanotrophs and methylotrophs should arise from a comparative study of their genomes and proteomes (Berven *et al*., 2006) that may clarify which of the genes/enzymes have been lost during evolution or are present but not expressed in obligate methanotrophs. Paradoxically, a

complete genomic analysis of *Mc. capsulatus* Bath (3.3 Mb, 3120 ORFs) (Ward *et al*., 2004) and partial genome of *Methylomonas* sp. 16A (4.3 Mb, 4,000 ORFs) (Sharpe *et al*., 2002) have led to surprising results. In both genomes, the genes coding E_1 and E_2 structural components of α-ketoglutarate dehydrogenase are present. Notably, theoretical considerations of the physicochemical properties of these gene products showed a high similarity with the active enzymic subunits of heterotrophic bacteria (Wood *et al*., 2004).

Very recently, the inability of the obligate methanol and methylamine utilizer *Methylobacillus flagellatum* KT to grow on multicarbon compounds was explained by an incomplete TCA cycle since no genes potentially encoding α-ketoglutarate-, succinate-, and malate-dehydrogenase were identifiable in the genome of this Betaproteobacterium (Chistoserdova *et al*., 2007). However, it is now recognized that such an explanation for all cases of obligate methylotrophy and autotrophy, that is, the absence of one or a few genes, is too simplistic (Wood *et al*., 2004). In fact, the C1 obligateness of *Mb. flagellatus* KT is due to a highly specialized C1 metabolism which is deficient in a whole module of genes/enzymes of glycolysis, the TCA cycle and the glyoxylate shunt (Kletsova *et al*., 1987).

Comparative analysis of the genomes of some obligately autotrophic cyanobacteria and Archaea confirmed the absence of at least one of the components of α-ketoglutarate dehydrogenase, although in these microorganisms a plausible ferredoxin-dependent mechanism for α-ketoglutarate decarboxylation to succinyl-CoA operates (Wood *et al*., 2004). The primary results raised some interesting questions. Why are the active pyruvate- or α-ketoglutarate dehydrogenases not synthesized, even though the appropriate encoding genes are retained in chromosomes of obligate methanotrophs? Is the absence of these and other functional genes/enzymes of central metabolism an initial reason/cause of C_1 obligateness? Indeed, comparative genomic sequencing and phylogenetic profiling has revealed a complicated metabolic and genetic organization and evolution of both obligate methanotrophs, methylotrophs and autotrophs (Chistoserdova *et al*., 2007; Ward *et al*., 2004; Wood *et al*., 2004). Nontheless, analyses of more genome sequences, including those from facultative methanotrophs such as *Methylocella silvestris* BL2, the genome of which is currently being sequenced, are necessary before these questions can be properly resolved.

V. CONCLUSIONS AND OUTLOOK

In this review, we summarized the current state of knowledge about the unique metabolic organization of aerobic obligate methanotrophic bacteria utilizing methane as a carbon and energy source. Regulation of

the expression of soluble and particulate forms of methane monooxygenase is mediated by copper ions. In contrast to sMMO, many biochemical aspects of pMMO structure, mechanism, and regulation are still obscure. The involvement of the tetrahydrofolate- and tetrahydromethanopterin-dependent enzymes in activation and oxidation of formaldehyde to formate followed by the oxidation to CO_2 by formate dehydrogenase, as well as the coupling of most of these enzymes to electron transport chain via cytochromes *b* and *c* is also a special feature of these ubiquitous bacteria. Central carbon metabolism of aerobic methanotrophs is carried out by the Quayle ribulose monophosphate or serine cyclic pathways correlating with intracytoplasmic membranes characteristic of type I and type II methanotrophs respectively. Also, the lack of activities of α-ketoglutarate dehydrogenase or the E_1 component of pyruvate dehydrogenase have been observed, despite the presence of the genes encoding these enzymes. Several other enzymic lesions in the central carbon metabolism of obligate methanotrophs (glyoxylate bypass and gluconeogenesis) have been found. Type I methanotrophs assimilate ammonia mainly by reductive amination of pyruvate/α–ketoglutarate, whereas type II methanotrophs employ the glutamate cycle. In general, such highly specialized structure–functional organization found in obligate methanotrophs may be a biochemical rationale for their strict dependence on CH_4 and inability to grow on multicarbon substrates. Further biochemical, genomic, and proteomic analyses of obligate and newly discovered facultative methanotrophs will give new insights into the molecular basis of obligate methanotrophy in these bacteria.

Finally, aerobic methanotrophs, discovered by Kaserer and Söhngen just over a century ago, are still enigmatic bacteria despite great advances in studies of their unique metabolic pathways. However, the advent of modern molecular, genomic, and proteomic methodologies will enable us to decipher phylogeny, metabolic regulation (adaptation), and genetic organization of various methanotrophs. These exciting prospects will be efficiently applied or exploited in modern biotechnology. The most promising in this respect are the extremophilic/thermotolerant methanotrophs such as *Mc. capsulatus* Bath. To date, this intriguing ''microbial Rosetta stone'' (Whittenbury, 1980) is the best studied methanotroph at the biochemical and molecular levels. Further work on deciphering the genetic and biochemical blueprint of the *Mc. capsulatus* genome will shed more light on the physiological and metabolic peculiarities of other methanotrophs living in the extreme environments and thriving in the presence of various stress-factors (Trotsenko and Khmelenina, 2002, 2005). Analogous questions concerning the simultaneous operation of several pathways of C_1-metabolism and the reasons for their activation/repression in diverse methanotrophs, including the newly discovered facultative methanotrophs of the genus *Methylocella* (Dedysh *et al*., 2005;

Theisen and Murrell, 2005; Theisen *et al*., 2005) and the recently reported filamentous facultative methanotrophs *Crenothrix polyspora* (Stoecker *et al*., 2006) and *Clonothrix fusca* (Vigliotta *et al*., 2007), will hopefully be resolved when more information about their genomes and proteomes are available in the near future. Last, but not the least, are the questions concerning the origin and evolution of the key enzymes/genes involved in (an)aerobic C1 metabolism: did the homologous genes evolve from a common ancestor, or were they transferred horizontally? Genomic sequencing has revealed a large number of fundamental reactions in the Bacteria and Archaea that are catalyzed by similar enzymes having high identity. Evidently, comparative analyses of more genomes and proteomes are required to answer the most intriguing question of the origin of these common genes/enzymes crossing bacterial/archaeal boundaries.

NOTE ADDED IN PROOF

The authors would like to draw the attention of readers to three exciting new papers on thermoacidophilic methanotrophs of the phylum Verrucomicrobia, which further extend the range of extant methanotrophs.

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CHAPTER 6

Bacterial Efflux Transport in Biotechnology

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Contents

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I. INTRODUCTION

The increasing need for and interest in environmentally friendly technologies using renewable feedstocks has lead to continuing developments in bioprocesses. Valuable chemicals can be produced by metabolic engineering of biosynthetic pathways or through one or more biotransformations. This synthetic biology requires numerous factors to be optimized for efficient biological production of chemicals. Among these the important considerations are uptake of the substrate molecule into the biocatalyst cell and efflux of the end product. In many cases, the genes and proteins responsible for uptake membrane transport are well known and thus can be readily optimized. In contrast, the systems involved in efflux membrane transport are not well defined. Accordingly, gene function discovery may be required as part of a program in bioprocess development.

Optimized efflux transport is an important issue for complex multistep conversions using engineered biosynthetic pathways and also for more simple one or two step bioconversions using whole cell catalysts. When microbial metabolic pathways are manipulated to produce molecules of interest, the transport of the final product out of the cell must be considered along with the rest of the pathway. Optimizing the expression of efflux transport in biocatalyst cells can lead to increased product yield, improved product tolerance of production organisms, and maximized product recovery from the extracellular medium. Likewise, in whole cell bioconversions, the tolerance of the biocatalyst to the process conditions, often in the presence of an organic solvent, as well as to the end product can be improved by genetic manipulation of efflux transport.

This review will focus on examples of bacterial efflux transport systems as they have been applied in synthetic biology. Much of the progress in discovery of efflux transport systems has been in the field of multidrug efflux pumps and there are numerous review articles that discuss these systems in gram negative bacteria (Poole, 2004, 2007; Zgurskaya and Nikaido, 2000) and gram positive bacteria (Kaatz and Dingell, 2005; Lorca *et al*., 2007; Markham and Neyfakh, 2001). Previous reviews on efflux transport systems in biotechnology include those on amino acid efflux systems (Burkovski and Kramer, 2002; Eggeling and Sahm, 2003) and on the role of efflux systems in solvent tolerance (Fernandes *et al*., 2003).

II. IMPORTANT EFFLUX TRANSPORT PROTEIN FAMILIES

Table 6.1 summarizes key families of bacterial efflux transport proteins for small organic molecules. These protein families are thus important for consideration in bioprocesses development.

TABLE 6.1 Important protein families for efflux transport of small organic molecules

Family name	Abbreviation	TC classification	Energy source	Example
Aromatic acid exporter family	ArAE	2.A.85	PMF	*Escherichia coli* AaeB
ATP-binding cassette superfamily	ABC	3.A.1	ATP	*Lactococcus lactis* LmrA
Autoinducer-2 exporter family	AI-2E	2.A.86	?	*Escherichia coli* TqsA
Branched chain amino acid exporter family	LIV-E	2.A.78	PMF	*Corynebacterium glutamicum* BrnEF
Drug/metabolite transporter superfamily	DMT	2.A.7	PMF	*Staphylococcus aureus* Smr
L-Lysine exporter family	LysE	2.A.75	PMF	*Corynebacterium glutamicum* LysE
Major facilitator superfamily	MFS	2.A.1	PMF	*Bacillus subtilis* PbuE
Multidrug/oligosaccharidyl-lipid/polysaccharide flippase superfamily	MOP	2.A.66	Na ion gradient	*Vibrio parahaemo-lyticus* NorM
Resistance to homoserine/threonine family	RhtB	2.A.76	PMF	*Escherichia coli* RhtB
Resistance-nodulation-cell division superfamily	RND	2.A.6	PMF	*Pseudomonas putida* TtgH
Threonine/serine exporter family	ThrE	2.A.79	PMF	*Corynebacterium glutamicum* ThrE

A. Energy sources and physiological roles

The majority of these efflux proteins use proton motive force (PMF) or transmembrane sodium gradients as energy source to catalyze transport, with a typical reaction:

$$\text{Substrate (in)} + \text{nH (out)} \rightarrow \text{Substrate (out)} + \text{nH (in)}$$

The large ABC superfamily of efflux proteins uses energy from ATP directly:

$$\text{Substrate (in)} + \text{ATP} \rightarrow \text{Substrate (out)} + \text{ADP} + \text{Pi}$$

It is important to note that some of the protein families listed in Table 6.1 include members that catalyze both uptake and efflux transport (ABC, DMT, and MFS), while the other protein families are likely specific for efflux transport (ArAE, AI-2E, LIV-E, LysE, MOP, RhtB, RND, and ThrE). The physiological role of efflux transport in bacteria may range from protection of the cell from toxic compounds found in the environment to restoring cellular homeostasis following metabolite imbalances resulting from normal cellular processes. The latter function has been termed a metabolic relief valve (Van Dyk *et al*., 2004) and may include amino acids built up from overflow metabolism (Kramer, 1994), excess intermediates of biosynthetic pathways (Van Dyk *et al*., 2004), or mutagenic compounds generated during metabolism (Gabrovsky *et al*., 2005). Another physiological role for efflux transport is in intercellular signaling (Yang *et al*., 2006). Specific quorum sensing molecules, such as autoinducer 2 (Herzberg *et al*., 2006) and homoserine lactones (Chan *et al*., 2007), are effluxed. Furthermore, it is proposed that other cellular metabolites may be exported and used as signaling molecules (Zakataeva *et al*., 2006).

B. Functions in gram negative and gram positive bacteria

Most of the protein families in Table 6.1 are found in both Gram negative and Gram positive bacteria. However, in Gram negative bacteria several of the efflux protein families (ArAE, ABC, MFS, RND) work with accessory proteins to pump substrates past the inner and outer membranes (Fig. 6.1). These accessory proteins include periplasmic proteins in the membrane fusion protein family (MFP) and the channel-tunnel proteins that span the periplasmic space and outer membrane in the outer membrane factor family (OMF). The resultant tri-partite efflux systems have the advantage of pumping hydrophobic substrates past the outer membrane, which is an excellent permeability barrier for hydrophobic molecules (Nikaido, 2003). Thus, gram negative bacteria have the synergistic action of a permeability barrier and active efflux for defense against toxic compounds. Most gram positive bacteria, on the other hand, have only efflux systems for removal of compounds that are deleterious to cell growth or viability (Fig. 6.1). However, some Gram positive bacteria, such as the *Corynebacterium–Nocardia–Mycobacterium* group, have a lipid-rich cell wall that functions as a pseudo outer membrane with permeability barrier properties (Nikaido, 2003).

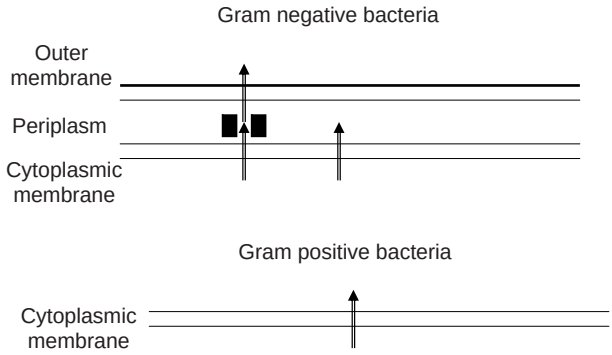

FIGURE 6.1 Schematic drawing of efflux transport systems in Gram negative and Gram positive bacteria. The thick solid line, the outer layer of the outer membrane of Gram negative bacteria, represents the effective permeability barrier. Efflux across both the cytoplasmic and outer membrane is accomplished by a tri-partite efflux system comprising an inner membrane efflux pump, a periplasmic component from the MFP protein family, and channel-tunnel protein that spans the periplasmic space and the outer membrane from the OMF family. Gram negative bacteria also have efflux systems that pump substrates across the cytoplasmic membrane to periplasmic space. Gram positive bacteria lack an outer membrane and thus have efflux systems that pump substrates only across the cytoplasmic membrane.

C. Substrate specificity

Some of these protein families listed in Table 6.1 have broad substrate specificity and in many cases are responsible for a multidrug resistance (MDR) phenotypes. The substrate specificity of the MDR efflux pumps may include compounds of interest as endproducts of metabolic engineering. However, efflux systems with narrower substrate specificity are typically the more relevant class for synthetic biology. Critical cell metabolites are substrates for some MDR pumps (Helling *et al.*, 2002) and thus optimizing efflux of the desired product without disrupting cellular metabolism, which may be difficult with these multisubstrate efflux transporters. On the other hand, efflux transporters that include the desired endproduct in a limited substrate range are far more useful for metabolic engineering because the compounds effluxed from the cell can be limited to the compounds of interest to be produced by bioprocesses.

D. Internet resources

Two useful resources for analyses of membrane transport proteins are the TC classification system (http://www.tcdb.org/), a comprehensive classification system for membrane transport proteins (Saier *et al.*, 2006),

and TransportDB (http://www.membranetransport.org/), for genomic comparison of transport proteins (Ren *et al*., 2007). The TC system is analogous to the Enzyme Commission (EC) system for classification of enzymes, with the exception that both functional and phylogenetic information are included. The first component of the TC classification (a number) corresponds to the transporter class (i.e., channel, carrier (porter), primary active transporter, or group translocator). The second component (a letter) corresponds to the transporter subclass which, in the case of primary active transporters, refers to the energy source used to drive transport. The third component (a number) corresponds to the transporter family or superfamily. The fourth component (a number) corresponds to the subfamily in which a transporter is found, and the final component corresponds to the substrate or range of substrates transported. Table 6.1 lists the first three components of the TC classification for the known protein families involved in small organic molecule efflux.

III. DISCOVERY OF EFFLUX TRANSPORT FUNCTION

Although many efflux transport systems have been described, it is not very likely that all the families of efflux transporters are currently known. Furthermore, within a family of efflux transporters, substrate specificity and the range of substrates transported are often not well defined.

A. Global gene expression analyses

The need for gene function discovery will often arise in a metabolic engineering program. For example, microbial production of para-hydroxybenzoic acid (pHBA) for applications such as liquid crystal displays is of interest (Barker and Frost, 2001; Miller and Peretti, 2002; Ramos-Gonzalez *et al*., 2003). Accordingly, there was a need to understand efflux of pHBA from cells engineered to produce it. One approach to discovery of a pHBA efflux system was to treat *Escherichia coli* cultures with pHBA and examine global gene expression changes with DNA microarrays. The underlying assumption in this approach was that if pHBA had toxic effects on the cell, the defense systems will be upregulated and these defense systems may include efflux systems. These expression studies led to the identification of candidate cotranscribed *E. coli* genes encoding proteins from the MFP family, AaeA, and the ArAE family, AaeB. These genes were subsequently shown to encode an efflux system of quite narrow substrate specificity that includes pHBA (Van Dyk *et al*., 2004). Prior to these discoveries, there was no known function for any member of the ArAE family, which had previously been described as a putative efflux family based on bioinformatic analysis

(Harley and Saier, 2000). Genome wide expression profiling under the conditions of elevated cytoplasmic concentrations of L-methionine was also used for the discovery of a LIV-E family, BrnEF, L-methionine efflux system in *Corynebacterium glutamicum* (Trotschel *et al*., 2005). Interestingly, in this case the BrnEF efflux system had previously been shown to efflux L-isoleucine, but L-methionine is likely the native substrate because it is a better inducer of *brnEF* expression.

B. Genetic selections and screens

Other approaches to efflux function discovery include genetic selections or screens under conditions of elevated cytoplasmic concentrations of the molecules of interest. For example, feeding the dipeptide Lys-Ala to *Corynebacterium glutamicum* increases the cytoplasmic concentration of lysine so that strains lacking a functional LysE L-lysine efflux system cannot grow on plates containing this peptide (Vrljic *et al*., 1996). This observation was exploited to discover *C. glutamicum* isoleucine and L-threonine efflux systems by screening transposon mutant collections for strains hypersensitive to peptides containing these amino acids (Kennerknecht *et al*., 2002; Simic *et al*., 2001). In a related way, an *E. coli* RhtB family protein (LeuE) for L-leucine efflux system was discovered by overexpression of the gene conferring resistance to glycyl-L-leucine (Kutukova *et al*., 2005). Furthermore, overexpression of *leuE* in L-leucine producing strains resulted in elevated production of L-leucine.

In addition to searching for specific efflux systems, broader phenotypic screens using collections of mutants or overexpression clones have been conducted. A set of *E. coli* mutants in predicted MDR efflux genes were screened for alterations in antibiotic sensitivity (Sulavik *et al*., 2001), as were a group of *E. coli* strains carrying multicopy clones of many predicted efflux systems (Nishino and Yamaguchi, 2001), leading to insights on substrate specificity for numerous efflux pumps. Similarly, a broad screen for efflux functions related amino acids was conducted using *E. coli* mutants and overexpression clones that were tested for alterations in sensitivity to various amino acids and amino acid analogs (Rybak *et al*., 2004).

IV. ENGINEERING EFFLUX TRANSPORT TO IMPROVE AMINO ACID PRODUCTION

Several amino acids are commercially manufactured at large scale in bioprocesses using engineered bacterial strains. Much progress has been made in understanding systems for efflux of commercially important amino acids and in many cases the fundamental knowledge has been

applied to improve production strains such that higher titers and yields of amino acids were obtained. Some examples of discoveries of efflux transporters and the impact on the fermentative production of amino acids are discussed here.

A. L-Lysine

L-lysine has been produced using *C. glutamicum* for more than 40 years with current annual production exceeding 600,000 tons (Pfefferle *et al*., 2003). Improvements to production strains by metabolic engineering included genetic manipulations of several of the pathway genes, such as pyruvate carboxylase, asparate kinase, dihydrodipicolinate synthase, and homoserine dehydrogenase. In addition to these enzymes, genetic engineering of the specific L-lysine efflux pump, LysE, has been a key to optimizing L-lysine production (Pfefferle *et al*., 2003). The discovery and cloning of *lysE* in 1996 (Vrljic *et al*., 1996) opened the door to genetic manipulation of L-lysine efflux. When LysE was originally described, it was not related to any known transporter and thus is the original member of the LysE protein family that has members found in many bacteria (Vrljic *et al*., 1999). Further characterization of *C. glutamicum* LysE showed that L-arginine is also a substrate and both amino acids are effluxed at about 0.75 nmol/min/mg dry wt (Bellmann *et al*., 2001). In *C. glutamicum, lysE* expression is regulated by LysG, encoded by a divergently transcribed gene. Interestingly, in addition to L-lysine and L-arginine, L-histidine and L-citrulline induce expression of *lysE* although they are not substrates for efflux by LysE (Bellmann *et al*., 2001). Recently, a mutant of the *C. glutamicum lysE* gene expressed in a L-lysine producing strain of *Methylophilus methylotrophus* was shown to improve L-lysine production by 10-fold (Gunji and Yasueda, 2006), demonstrating heterologous expression of an efflux transporter to improve amino acid production.

B. L-Threonine

L-Threonine is an essential amino acid produced commercially by microbial fermentation for use in animal feed supplementation (Debabov, 2003). Advances in understanding mechanisms of L-threonine efflux and subsequent improvements in production have been obtained in both *C. glutamicum* and *E. coli*. The ThrE threonine efflux protein of *C. glutamicum* was initially defined by a transposon mutant hypersensitive to L-threonine containing peptides and was then shown to export both L-threonine and L-serine (Simic *et al*., 2001). When discovered, ThrE was the prototype of a new transporter protein family defined with homologues in select bacteria, archaea, and fungi (Yen *et al*., 2002).

Overexpression of *thrE* in a *C. glutamicum* strain that is defective for L-threonine degradation resulted in a 40% increase in final L-threonine titer (Simic *et al*., 2002). In *E. coli*, it was found that amplification of *rhtB* conferred resistance to homoserine and homoserine lactone, while amplification of *rhtC* provided resistance to L-threonine (Zakataeva *et al*., 1999). These two proteins are in different protein family from ThrE and were the prototype members of the RhtB family (Aleshin *et al*., 1999), which is part of the LysE superfamily of efflux proteins (Vrljic *et al*., 1999). Overexpression in *E. coli* L-threonine producing strains of the *E. coli rhtB* or *rhtC* or *C. glutamicum thrE* markedly improved (up to 290%) L-threonine production of a strain producing 8 g/liter L-threonine and also substantially improved (up to 80%) L-threonine production in a more efficient producer (Kruse *et al*., 2002). Interestingly, overexpression of *E. coli rhtA*, encoding another L-threonine efflux system from a third protein family, the DMT superfamily, increases production of L-threonine, L-lysine, L-proline, and homoserine by respective producing strains (Livshits *et al*., 2003b).

C. L-Phenylalanine

L-Phenylalanine is commercially produced by microbial fermentation in large quantities for various applications, the most significant of which is as a component of aspartame, an artificial sweetener (Grinter, 1998). Although there are numerous publications on metabolic engineering of the L-phenylalanine biosynthetic pathway in various microorganisms, in particular *E. coli*, not much is known about the efflux transport of this amino acid. Nonetheless, L-phenylalanine efflux is considered of critical importance to the large scale fermentation production in part because L-phenylalanine is only recovered from the extracellular medium and washed cells, thus up to 5% of the total production may be lost in cell biomass (Fotheringham, 2000). Metastable *E. coli* mutants have been isolated that hyper-export L-phenylalanine; although the specific efflux mechanism was not identified, conditions to stabilize the phenotype were found (Grinter, 1998). More recently, a hint about L-phenylalanine efflux has come from selections for resistance to L-phenylalanine and other amino acid analogs. Multicopy expression of a native *E. coli* gene, *yddG*, in *E. coli* conferred resistance in these selection conditions and enhanced production of L-phenylalanine and L-tryptophan (Livshits *et al*., 2003a). The product of *yddG* is a membrane protein with 10 transmembrane helices in the DMT family and thus is likely to be a transport protein. Furthermore, the closely related YddG protein of *Salmonella enterica* sv. Typhimurium is involved in methyl viologen efflux (Santiviago *et al*., 2002). Thus, it is likely that YddG is an efflux system for methyl viologen, L-phenylalanine, and other aromatic compounds.

D. L-Cysteine

In contrast to the amino acids mentioned earlier, L-cysteine is primarily produced by protein hydrolysis, but recent advances combining enhanced biosynthetic activity, decreased degradation, and efficient efflux systems have resulted in effective microbial fermentation production (Wada and Takagi, 2006). Progress in understanding mechanisms of L-cysteine efflux is most advanced in *E. coli*. A plasmid-borne chromosomal library of *E. coli* in an L-cysteine producing *E. coli* strain screened for increased L-cysteine production yielded a clone carrying the gene *ydeD* (now called *eamA*) (Dassler *et al*., 2000). Characterization of EamA, a protein in the DMT superfamily, revealed that it was an efflux protein for L-cysteine and *O*-acetyl-L-serine. Likewise, overexpression of *E. coli yfiK* (now called *eamB*) lead to increased L-cysteine production in an industrial *E. coli* L-cysteine producing strain (Franke *et al*., 2003). EamB like EamA is also an efflux protein for L-cysteine and *O*-acetyl-L-serine but unlike EamA is the RhtB protein family. It has also been reported that in *E. coli* Bcr, a multidrug efflux pump in the MFS protein family will also efflux L-cysteine, resulting in increased production (Yamada *et al*., 2006). Yet another *E. coli* L-cysteine efflux system, CydDC, in the ABC protein family has been reported (Pittman *et al*., 2002), but has not been exploited for improvement of L-cysteine production. However, the *ydeD* (*eamA*) encoded efflux system has been applied in a two step process to produce unnatural L-amino acids (Maier, 2003). The first step, production of *O*-acetyl-L-serine, was accomplished with an *E. coli* strain carrying a plasmid with both $cysE^{fbr}$ and *ydeD* and yielded 9 grams/liter. An *E. coli* strain carrying $cysE^{fbr}$ but not *ydeD* produced no detectable amounts of *O*-acetyl-L-serine. Thus, the success of this bioprocess was critically dependent on genetic manipulation of an efflux system.

V. EFFLUX TRANSPORT IN WHOLE CELL BIOTRANSFORMATIONS

Efflux transport is also important for biotransformations or bioconversions in which whole cells are used to catalyze one or more chemical reactions.

A. Solvent tolerant bacteria

It is often desirable to conduct such biotransformations in a two phase system for *in situ* removal of toxic products or to limit aqueous concentrations of toxic substrate molecules. Accordingly, bacteria tolerant to the organic solvents used as the second phase are being actively developed

for these biotransformations, which are expected to soon compete economically with chemical synthesis (Heipieper *et al*., 2007). The mechanisms of tolerance to toxic organic solvents such as toluene and 1-decanol have been intensively studied in solvent tolerant strains of *Pseudomonas putida* S12 (Isken and De Bont, 2000; Weber *et al*., 1993) and DOT-T1E (Ramos *et al*., 2002; Ramos *et al*., 1995). The adaptive mechanisms of solvent tolerance involve membrane modifications and active efflux for removal of solvent molecules from cytoplasmic membranes.

In *P. putida* DOT-T1E, three efflux pumps, each in the RND protein family, contribute to solvent resistance (Rojas *et al*., 2001). Each of the genes encoding the RND family efflux protein are found in operons with accessory proteins from the MFP and OMF families for transport out across both the cytoplasmic and outer membranes. Of these three three-component systems, the one encoded by *ttgGHI* is found to be more quantitatively important for solvent tolerance than those encoded by *ttgABC* or *ttgDEF* (Rojas *et al*., 2001). Recently, the *ttgGHI* efflux pump operon was found to be located on a large, very stable, self-transmissible plasmid (Rodriguez-Herva *et al*., 2007). Several biotransformation processes are being developed using *P. putida* DOT-T1E to capitalize on its solvent tolerance (Meyer *et al*., 2005; Neumann *et al*., 2006; Neumann *et al*., 2005; Ramos-Gonzalez *et al*., 2003).

In *P. putida* S12, an RND family efflux pump is also responsible for solvent tolerance and the operon encoding it, *srpABC*, also encodes an MFP family protein and an OMF family protein (Kieboom *et al*., 1998). Another RND family efflux pump and associated accessory proteins encoded by the *arpABC* operon is present in *P. putida* S12, but is not involved in solvent tolerance (Kieboom and de Bont, 2001). *P. putida* S12 has been used as a host strain for various bioprocesses and biotransformations that take advantage of its solvent tolerance (Nijkamp *et al*., 2005, 2007; Wery *et al*., 2000; Wierckx *et al*., 2005).

B. Mitigation of substrate and product toxicity

Efflux systems are also important to mitigate toxicity of the substrates and end products of biotransformations. For example, in the bioconversion of toluene to pHBA developed in *P. putida* DOT-T1E both the substrate and product are toxic molecules (Ramos-Gonzalez *et al*., 2003). Strain DOT-T1E was originally isolated as a toluene tolerant strain and the efflux pumps described earlier are involved in toluene tolerance. This strain was also found to tolerate high concentrations of pHBA and while not characterized, energy-dependent efflux systems play a role in removal of pHBA from cell membranes (Ramos-Gonzalez *et al*., 2001).

VI. LIMITS ON EFFLUX TRANSPORT UTILITY IN METABOLIC ENGINEERING

A. Hydrophobicity considerations

The degree of hydrophobicity of the compound to be produced defines whether efflux transport should be considered in developing a synthetic biological process. The examples mentioned earlier demonstrate the importance of efflux transport for the production of relatively hydrophilic molecules, amino acids, and for tolerance to relatively hydrophobic molecules, organic solvents. Amino acids and other relatively hydrophilic molecules will be excluded from re-entry to the cell by the cytoplasmic membrane. Accordingly, efflux transport across one membrane is effective in preventing a futile cycle of efflux and re-entry, assuming that uptake systems for the compounds are not active. More hydrophobic molecules, such as toluene or 1-decanol, will freely diffuse through the cytoplasmic membrane, thus efflux systems that confer resistance to these molecules pump them out past both the cytoplasmic and outer membrane of gram negative bacteria. The lipopolysaccharide layer of the asymmetric outer membrane is then a permeability barrier inhibiting the re-entry of these molecules and thus limiting futile cycling. What about compounds of intermediate hydrophobicity, for example ethanol or 1,3-propanediol? In these cases, efflux transport may not be useful. As in the case of more hydrophobic molecules, the cytoplasmic membrane will not be an effective permeability barrier. However, unlike the situation with the more hydrophobic compounds, the outer membrane does not serve as a permeability barrier because nonspecific porins allow passage of such molecules as they do for compounds required for cell growth (Nikaido and Vaara, 1985). Thus, genetic engineering of cellular metabolism to produce compounds such as 1,3 propanediol can be accomplished without concern for efflux transport (Nakamura and Whited, 2003).

B. Availability of known transporters and protein engineering

Another limit on the utility of efflux transport for metabolic engineering is the availability of known transporters for the compounds of interest. In the genomes of every bacteria that has been sequenced there are numerous predicted efflux transporters for which the physiological substrates are not known. Thus, gene function discovery may be required to uncover the needed transporter gene. Such discoveries are particularly likely if the compound is related to a normal cellular metabolite or an environmental compound naturally encountered by the bacteria. Starting with an efflux transporter with some activity for the compound of interest, protein engineering or directed evolution can be applied to

improve the substrate specificity or activity of the transporter (Soberon and Saier, 2006). One example where this has been accomplished is the TetA tetracycline efflux transporter, where mutations in a cytoplasmic loop reduced tetracycline resistance and increased the relative resistance to doxycycline and minocycline, indicating a change in substrate specificity (Sapunaric and Levy, 2005).

VII. FUTURE PROSPECTS FOR EFFLUX TRANSPORT IN BIOTECHNOLOGY

Discovery of efflux transport system functions is continuing at a rapid pace as researchers study multidrug resistance, solvent tolerance, and production of small molecules by synthetic biology. As more and more systems are described, the ongoing need for gene function discovery will decrease. However, as efflux transport is continued to be applied in biotechnology, there will be increasing application of protein engineering to modify or enhance functions. In conjunction with improvements in pathway metabolic engineering, genetic manipulation of bacterial efflux transport will play an ongoing role in strain development for commercial bioprocesses using renewable resources for production of needed chemicals.

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CHAPTER 7

Antibiotic Resistance in the Environment, with Particular Reference to MRSA

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I. INTRODUCTION

The introduction of β-lactam antibiotics (penicillins and cephalosporins) in the 1940s and 1950s probably represents the most important event in the battle against infection in human medicine. Even before widespread global use of penicillin, resistance was already recorded. *E. coli* producing a penicillinase was reported in Nature Journal in 1940 (Abraham and chain, 1940) and soon after a similar penicillinase was discovered in *Staphylococcus aureus* (Kirby, 1944). The appearance of these genes, so quickly after the discovery and before the widespread introduction of penicillin, clearly shows that the resistance genes pre-dated the clinical use of the antibiotic itself.

Intuitive reasoning would suggest that antibiotic resistance occurs because of direct selection produced by the use of antibiotics in humans and animals. For example, the mutations associated with increased resistance to fluoroquinolones have been documented in specific regions of the *gyrA, gyrB, grlA,* and *grlB* genes, which are referred to as the quinolone resistance-determining regions (QRDRs) (Piddock, 1998). Selection for resistance to a given antibiotic may take place within an infected human treated with antibiotics. However, selection may occur in other environments such as waste water treatment systems, agricultural environments where antibiotics may be of veterinary origin, or within an environmental background where antibiotic selection is provided by bacterial antibiotic producers.

In contrast to the scenario where resistance is conferred by mutation and selection by medical antibiotics, resistance can occur in an organism by the acquisition of a novel gene. New genes are acquired by horizontal gene transfer (HGT), through conjugation, transformation, or transduction. The origins of mobile antibiotic resistance genes may be from bacteria that have been subject to antibiotic selection in a nosocomial environment, or from environmental bacteria.

An example of an environment where HGT is likely to occur is soil. Practices such as sewage sludge and animal slurry application introduce complex mixtures of bacteria containing drug resistance genes, medical and veterinary antibiotics, and other chemicals such as detergents and surfactants to land, where interactions may occur with indigenous soil bacteria (Fig. 7.1).

II. EVOLUTION OF RESISTANCE

Antibiotic resistance has two components: the evolution of genes with novel activities and the evolution of mechanisms allowing horizontal transfer throughout the microbial population.

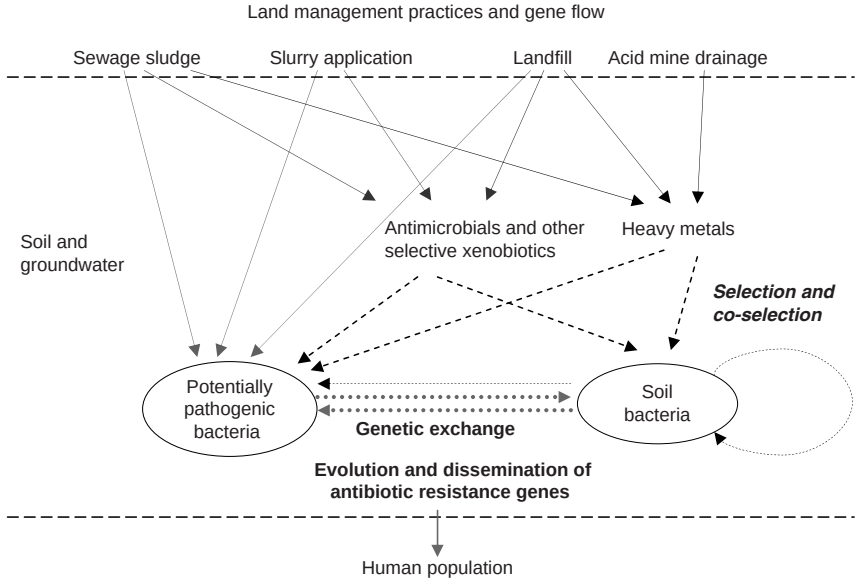

FIGURE 7.1 Anthropogenic sources of bacterial pathogens, pharmaceuticals, and heavy metals, which, in conjunction with indigenous soil bacteria, provide a mixture of genes and selective pressure for selection or coselection of antibiotic resistance.

A. Origins of antibiotic resistance genes

Many modern β-lactam antibiotics such as the 7-α-methoxycephalosporins are secondary metabolites of *Streptomyces* species; the majority of streptomycetes and other actinobacteria produce constitutively expressed β-lactamases, which have a high GC content (≥70%). However, the β-lactamases encountered in human and animal pathogens all have GC contents in the order of 45–65%, suggesting a Gram negative origin. There are several Gram negative bacteria that exist in close proximity to antibiotic producers in the rhizosphere, which calls for protection against the toxic metaboloites of their neighbors. Certain plant pathogens and rhizobacteria such as *Erwinia, Serratia, Flavobacterium, Pseudomonas, Chromobacterium* and *Agrobacterium* sp. produce carbapenems, β-lactams, and moncocyclic β-lactams (Jensen and Demain, 1995).

The rhizosphere is the soil compartment influenced by plant root metabolism, and is high in nutrients derived from root exudates consisting of compounds such as organic acids, sugars, amino acids, vitamins, and carbohydrates. The rhizosphere is an important niche for microorganisms involved in nutrient recycling and plant health. The resistance genes in the rhizosphere are likely to result from competition between microrganisms for colonization sites. Key mechanisms responsible for the selection of medically significant bacteria in the rhizosphere are discussed

in a recent review (Berg *et al*., 2005). Many mechanisms involved in the interaction between antagonistic plant-associated bacteria and their host plants are similar to those responsible for bacterial pathogenicity, including pathogenicity in humans (Rahme *et al*., 1995).

It has long been suspected that the environment constitutes a reservoir of novel antibiotic resistance genes, although its significance has been overlooked in favor of evolution of resistance within the clinical environment. Arguably one of the most clinically important groups of β-lactamases in Gram negative bacteria at the moment are the CTX-M family (Livermore and Hawkey, 2005). The identification of environmental progenitors of the extended-spectrum β-lactamase (ESBL) CTX-M enzymes, responsible for resistance to 3rd generation cephalosporins (3GCs), in bacteria of the genus *Kluyvera* clearly indicates the significance of the environment in the evolution of emerging antibiotic resistance determinants (Bonnet, 2004; Rodriguez *et al*., 2004). *Kluyvera* sp. are rare human pathogens causing infections similar to *E. coli* and are more often found associated with plants. *K. ascorbata* has been shown to enhance plant growth, particularly in heavy metal contaminated soils (Burd *et al*., 1998, 2000). *K. georgiana* produces KLUG-1, whose nucleic acid sequence clusters with the CTX-M-8 group (Fig. 7. 2). Sequence similarity between the genes suggests that the natural β-lactamases of *K. ascorbata* and *K. georgiana* are the progenitors of the CTX-M-2 and CTX-M-8 groups, respectively (Bonnet, 2004). Evidence suggests that the process of gene transfer from the chromosome of *Kluyvera* to other clinically important bacteria has occurred several times involving different mobile elements, such as the IS-10-like element found upstream of both KLUG-1 and CTX-M-8, and ISEcp1 found upstream of KLUA-1 and members of the CTX-M-2 group (Poirel *et al*., 2001). ESBLs confer low level resistance to β-lactams, *K. cryocrescens* possessing the KLUC-1 ESBL conferred resistance only to cefotaxime, ceftriaxone, cefpirome, and aztreonam when cloned into *E. coli* (Decousser *et al*., 2001). It is probable that KLUC-1 is only weakly expressed in *K. cryocrescens,* but mutations in the promoter region would confer ESBL resistance. Biochemical analysis of KLUC-1 revealed that substrate specificity and substrate profile are similar to those reported for CTX-M enzymes.

ESBLs confer resistance to 3GCs, which are semisynthetic molecules that do not exist in nature as far as is known; however, their structure is basically that of a cephalosporin of which a number of naturally produced compounds exist in the environment. Very little is understood about the prevalence, ecological function, and diversity of ESBL genes in the horizontal gene pool in soil, whether they are in transiently resident human/animal derived bacteria, or in the chromosomes of bacteria permanently residing in the rhizosphere.

A fluoroquinolone resistance gene *qnrA* has recently been established as originating in the water-borne species *Shewanella algae*; this genetically mobile plasmid-borne gene has now moved into clinically important bacteria, which

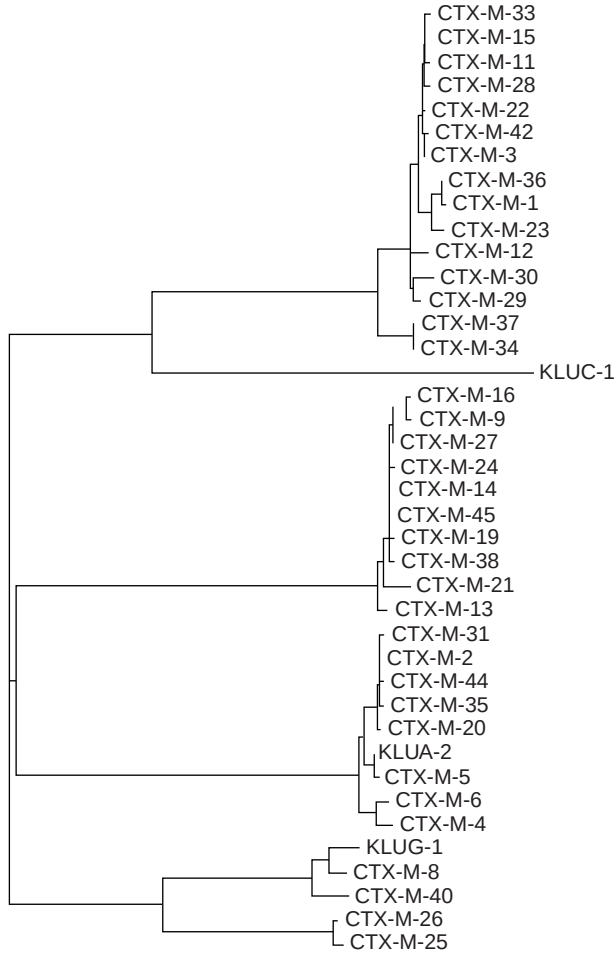

FIGURE 7. 2 Unrooted tree illustrating the phylogenetic relationships of KLUC-1, KLUG-1, and KLUA-1, *Kluyvera* sp. chromosomal genes, which are the putative progenitors of the CTX-M-1, CTX-M-8, and CTX-M-2 groups of ESBL enzymes (neighbor joining tree, Trex).

again shows the importance of the natural environment as a reservoir of clinically important antibiotic resistance genes (Nordmann and Poirel, 2005). The *qnrA* genes are embedded in complex sul1-type integrons, which also carry CTX-M-2 and CTX-M-9 ESBLs.

B. Mechanisms of resistance

There are four main types of antibiotic resistance in bacteria (Hawkey, 1998). Antibiotic modification allows retention of the same target as sensitive strains, but the antibiotic is modified before it reaches the target.

β-lactamases enzymatically cleave the β-lactam ring, inactivating the antibiotic. Some bacteria either stop the antibiotic from entering into the cell or pump the compound out of the cell by efflux. Carbapenem β-lactam antibiotics enter Gram negative bacteria via a membrane protein known as porin, but resistant bacteria lack the specific D2 porin responsible for transport and are therefore resistant (Pirnay *et al*., 2002). Efflux via a membrane pump is a common mechanism found in Gram negative and Gram positive bacteria, in which five different superfamilies of efflux pumps conferring antibiotic resistance have been reported (Mahamoud *et al*., 2007).

Changes in the target site also produce resistance; the antibiotics are able to reach the target but are not able to inhibit the target because of changes in the molecule. Enterococci are regarded as inherently resistant to cephalosporins because the enzymes responsible for cell wall synthesis (peptidoglycan production), known as penicillin binding proteins (PBPs), have a low affinity for cephalosporins and are not inhibited (Hawkey, 1998). Resistance to β-lactam antibiotics in pneumococci is entirely due to the development of altered forms of the high-molecular-weight PBPs, which have decreased affinity for the antibiotics (Coffey *et al*., 1995). Altered PBPs have emerged by recombinational events between the *pbp* genes of pneumococci and their homologs in closely related streptococcal species.

The fourth antibiotic resistance mechanism (usually an enzyme) is the production of an alternative target that is resistant to the antibiotic, whilst continuing production of the sensitive target. Methicillin resistant *Staphylococus aureus* (MRSA) produces an alternative penicillin binding protein (PBP2a), which is encoded by *mecA* carried on the Staphylococcal Cassette Chromosome mec (SCCmec). Because PBP2a is not inhibited by the antibiotics, the cell continues to produce peptidoglycan and maintains a stable cell wall (Hardy *et al*., 2004).

III. MECHANISMS OF HORIZONTAL GENE TRANSFER

Gene transfer in the environment is central to the hypothesis that a reservoir of novel resistance genes exists outside the clinic, which can be transferred to clinically significant bacteria in hospitals. An extensive literature on genetic exchange between bacteria in the environment exists, which is reviewed elsewhere (Davidson, 1999). The current review concentrates specifically on gene transfer mediated by transposable elements such as class 1 integrons, which are of increasing clinical importance. The occurrence of coselection by nonantibiotic xenobiotics is also discussed.

The evolutionary response of bacteria exposed to antibiotics has been mediated in large part by the movement of conjugative plasmids carrying

antimicrobial resistance genes in both Gram negative and Gram positive bacteria. Transfer frequencies for conjugative plasmids in *Enterococcus faecium* can be as low as $\geq 10^{-6}$ per donor in laboratory experiments using broth mating, but the production of short peptides, by some recipient cells, that cause cell aggregation greatly increases transfer rates ($\geq 10^{-4}$) (Dunny *et al*., 2001). Plasmids play an important role in gene transfer in staphylococci; conjugative plasmids are also capable of mobilizing small nontransferable plasmids (McDonnell *et al*., 1983). Gene transfer does not always require the presence of plasmids, as the extensively studied and widely distributed conjugative transposons such as Tn916 demonstrate (Burrus and Waldor, 2004). The gut of both humans and food animals is an important site for transfer of conjugative plasmids, as careful studies have shown there is no loss of fertility of conjugative plasmids in the gut. Conjugation probably occurs continuously in the gut of humans/animals even in those not receiving antibiotics (Freter *et al*., 1983). The release of faeces into the environment represents one of the most important sources of novel assortments and types of resistance one of genes in new bacterial hosts. The laboratory simulation of plasmid transfer in the environment can give low transfer frequencies, but these must be compared to the size of the populations involved. The requirement for close contact in the laboratory (e.g., filter mating) may readily be met in microenvironments: staphylococci on the skin, oral streptococci in dental plaque, or pseudomonads in water films on soil particles.

A. The role of integrons in resistance gene mobility

In addition to multiresistance plasmids, antibiotic resistance genes are situated on transposable elements that can associate with other elements such as chromosomes. These transposable elements include transposons and integrons, which can be transferred horizontally. Integrons are recombination and expression systems that capture genes as part of a genetic element known as a gene cassette (Recchia and Hall, 1995). Gene cassettes bear a recombination site known as a 59-base element (59-be) that is recognized by the integron-encoded integrase IntI (Hall *et al*., 1991). Most cassette genes described are antibiotic or quaternary ammonium compound resistance genes. However, recent studies have revealed that the cassette gene pool is far more diverse than previously thought. Stokes *et al*. (2001) designed PCR primers to conserved regions within the 59-be of gene cassettes, allowing detection of a large number of novel genes. Using these primers, 123 cassette types were recovered from Antarctic and Australian soils and sediments, with very few represented in clone libraries more than once indicating the large size of the cassette gene pool.

Most ORFs did not match known sequences, again illustrating the diversity of these gene sequences. Further studies revealed an additional 41 environmental gene cassettes, giving a total of 164 directly sampled from natural environments by PCR (Holmes *et al*., 2003). There are several classes of integron, the most commonly studied being class 1 integrons that are commonly associated with antibiotic resistant bacteria. Recent studies have detected novel integron classes in soils, Nield *et al*. (2001) classified three new integron classes and Nemergut *et al*. (2004) an additional 14 classes, demonstrating the immense variety of these elements present in the environment.

The variable regions of class 1 integrons contain the cassette genes, and to the right of this lies the 3′-conserved region, which may have one of three different backbone structures (Partridge *et al*., 2002). The first backbone type consists of a Tn402 (In16) like arrangement consisting of a *tni* module containing 3 transposition genes and a resolvase gene, the second In5 type consists of *qacE*Δ1, sul1, orf5, orf6, and a partial *tni* module (*tni*Δ) consisting of two transposition genes. The third In4 type just carries *qacE*Δ1, sul1, orf5, and orf6. Integrons carrying the complete *tni* module are able to undergo self-transposition and it is thought that In5 and In4 types may also be able to move if the *tni* gene products are supplied in *trans* (Partridge *et al*., 2002).

The role of class 1 integrons in conferring antibiotic resistance to clinical isolates of many bacterial strains is well documented (Briggs and Fratamico, 1999; Leverstein-van Hall *et al*., 2003; Segal *et al*., 2003; White *et al*., 2000). Fluit and Schmitz (2004) summarized recently described cassette gene diversity which includes 25 β-lactam resistance genes (including 8 carbapenemase and 17 ESBL genes), 11 aminoglycoside, 2 chloramphenicol, rifampicin, 3 trimethoprim, and quinolone resistance genes. It is therefore clear that integrons are capable of conferring resistance to extended-spectrum β-lactams, carbapenems and fluoroquinolones, representing an extremely efficient method of acquiring resistance to the most widely used and important antibiotics. Studies on the incidence of class 1 integrons in bacterial pathogens associated with agriculture and fish farming such as *E. coli* and *Aeromonas salmonicida* have shown a link between integrons and antibiotic resistance (Bass *et al*., 1999; Sorum *et al*., 2003). A recent study (Nemergut *et al*., 2004) illustrated evidence of a gene cassette encoding nitroaromatic catabolism, a group of compounds associated with mining activity, which highlights the fact that selective pressures other than antibiotics may also coselect for resistance genes. The process by which a gene becomes a movable cassette is not understood; however, it has been proposed that the 59-be is added as a transcription terminator to an RNA gene transcript, which is subsequently converted into DNA by a hypothetical reverse transcriptase (Recchia and Hall, 1995).

Class 1 and class 2 (also capable of carrying antibiotic resistance genes) are known to undergo transfer between bacteria in chicken litter, which is often spread onto soil where further selection and horizontal transfer may occur (Nandi *et al*., 2004). The integrons study was the first to demonstrate wide spread prevalence of class 1 integrons in Gram positive bacteria, illustrating the potential for HGT.

B. Coselection for resistance genes

It is commonly assumed that in the absence of antibiotic selection mobile resistance genes will be lost, and the host return to a sensitive phenotype, as the genes confer a metabolic cost on the host. Coselection is one mechanism whereby other resistance genes carried on the same genetic element produce selection for an entire mobile genetic element. Anthropogenic activity produces emissions of complex mixtures of xenobiotics, bacterial pathogens, and antibiotic resistance genes into the environment in the form of industrial and domestic effluent as well as human and animal waste. Industrial and domestic pollutants such as quaternary ammonium compounds (QACs) have been shown to exert an extremely strong selective pressure for class 1 integrons, which are a major mechanism for dissemination of antibiotic resistance (Gaze *et al*., 2005). Coselection is produced by the presence of QAC resistance genes on multiresistance plasmids or class 1 integrons. QAC resistance genes fall into two families: *qacA/B* belong to the Major Facilitator Superfamily and are only found in staphylococci on multiresistance plasmids (Paulsen *et al*., 1996). Other QAC resistance genes belong to the Small Multidrug Resistance Family and include *qacC/D* now known as *smr*, *qacE*, *qacEΔ1*, *qacF*, *qacG*, *qacH*, and *qacJ* (Gaze *et al*., 2005). *QacE*, *qacEΔ1*, *qacF*, and *qacG* have been identified on integrons and the remaining genes on multiresistance plasmids in staphylococci. In a study investigating QAC pollution and class 1 integron prevalence, bacteria were isolated from a reed bed used to remediate effluent from a textile mill (Gaze *et al*., 2005). QAC resistance was higher in isolates from reed bed samples and class 1 integron prevalence was significantly higher in populations pre-exposed to QACs.

Exogenous plasmid isolation can be used to detect resistance genes in soil bacteria. This method allows capture of plasmids from the total bacterial fraction of an environmental sample without the necessity to culture the host organism. Smit *et al*. (1998) investigated mercury resistance plasmids in soil populations using exogenous isolation, and identified plasmids of 10–50 kb carrying resistance to copper, streptomycin, and chloramphenicol. These authors amended soil with mercuric chloride and found this to subsequently increase the recovery of resistance plasmids, highlighting the fact that heavy metals may coselect for antibiotic resistance in the environment. Plasmids have also been captured from

polluted soils and slurrys (Smalla *et al*., 2000; Top *et al*., 1994). The latter authors identified multiple antibiotic resistance genes from isolated plasmids. Some aspects of animal husbandry using heavy metal containing compounds select for antibiotic resistance genes in the environment, for example, the use of copper growth supplements in pigs (Hasman *et al*., 2006). In Denmark glycopeptides were banned in food animal production in 1995, following that ban the rate of glycopeptide resistance in *Enterococcus faecium* (GRE) did not change. The banning of macrolides in 1998 led to a significant fall in glycopeptide resistance, this was thought to be due to both antibiotic resistance genes being on the same plasmid and coselection by the continuing use of macrolides (Aarestrup, 2000). GRE continue to be prevalent in Danish pigs; the reason for this is now thought to be the linkage on the same plasmid of the copper resistance gene *tcrB* and macrolide/glycopeptide resistances. A feeding experiment using 175 mg Cu/kg in feed selected for GRE in piglets, whereas 6 mg/kg feed did not (Hasman *et al*., 2006).

IV. ANTIBIOTICS AND RESISTANCE GENES IN THE ENVIRONMENT

Human and animal wastes may contain antibiotics or active intermediates from human and veterinary medicines that may potentially increase antibiotic resistance selection in soil, in addition to introducing pathogens, which can exchange mobile genes with indigenous rhizosphere bacteria. Antibiotics retain their selective capabilities in the soil and are ultimately released to surface waters (Boxall *et al*., 2002). Certain plant pathogens and rhizobacteria such as *Erwinia, Serratia*, *Flavobacterium, Pseudomonas, Chromobacterium,* and *Agrobacterium* sp. produce carbapenems, β-lactams, and moncocyclic β-lactams (Jensen and Demain, 1995). In addition, streptomycetes and fungi produce a wide range of antibiotics.

A. Sewage sludge

The 2001 UK Sewage Sludge Survey showed that an average of 1,072,000 tonnes of dry solids per annum was produced in 1998–2000. The UK Department for Food and Rural Affairs (DEFRA) state that conventionally treated sludge has been subjected to defined treatment processes and standards that ensure at least 99% of pathogens have been destroyed. Enhanced treatment, originally referred to as ''Advanced Treatment,'' describes treatment processes capable of virtually eliminating any pathogens (99.9999%) that may be present in the original sludge. Conventionally treated sewage sludge cannot be surface spread if the land is intended for grazing; it must be deeply injected into the soil and

left for at least three weeks until it is grazed. Conventionally treated sewage can be applied to the surface of grassland, or for forage crops such as maize, which will then be harvested (no grazing allowed within the season of application). If applied to vegetable-growing land at least 12 months must have elapsed between treatment and harvest. When using enhanced treated sludges farmers must wait at least three weeks before grazing the animals or harvesting the forage crops, and at least 10 months before harvesting fruit and vegetable crops grown in direct contact with the soil and normally eaten raw (http://www.defra.gov.uk/farm/waste/sludge/index.htm). It is clear that the potential for transmission of resistance genes to food animals and vegetable crops exists.

A hydraulic, biokinetic, and thermodynamic model of pathogen inactivation during anaerobic digestion showed that a 2 log10 reduction in *E. coli* (the minimum removal required by the UK government for agricultural use of conventionally treated biosolids) is likely to challenge most conventional mesophilic digesters (Smith *et al*., 2005). UK regulations for pathogen removal are becoming more stringent, but the processes used to reduce bacterial indicator species numbers may have a quite different effect on resistance gene numbers. β-lactam and aminoglycoside resistance genes have been isolated by exogenous isolation from activated sewage in Germany, illustrating that final stage sludge is a source of antibiotic resistance genes (Tennstedt *et al*., 2005).

Crucially, although sewage sludge has been demonstrated to contain antibiotic resistance genes and pathogenic bacteria, the extent of this problem and the potential for transfer of resistance to soil bacteria and ultimately its effect on the human population is unknown. Sewage sludge also contains measurable concentrations of antibiotics which may continue to select for resistance in the soil. A study by Golet *et al.* (2003) suggested that sewage sludge is the main reservoir of fluoroquinolone (FQ) residues from waste waters and outlined the importance of sludge management strategies to determine whether most of the human-excreted FQs enter the environment. Field experiments of sludge-application to agricultural land confirmed the long-term persistence of trace amounts of FQs in sludge-treated soils and indicated a limited mobility of FQs into the subsoil. Where sewage treatment plants receive large amounts of effluent from hospitals, the problem of antibiotic residues and resistance genes in sewage sludge may be particularly significant. Persistence of FQs is particularly relevant as they appear to coselect for class 1 integrons and integron borne ESBL genes. The recent discovery of new quinolone resistance genes (Nordmann and Poirel, 2005), which are situated on class 1 integron structures and also confer ESBL resistance, reinforces this. Our own research has indicated an impressive reservoir of FQ resistance

(QRDR mutations) in staphylococci associated with free range chicken farming (Hawes, 2004).

Recent studies in Portugal have identified β-lactamases, including TEM, IMP, and OXA-2 derivatives in aquatic systems, and ESBL resistance genes in sewage sludge, which is spread to land in the UK and therefore has the potential to recycle resistance to the human food chain (Henriques *et al*., 2006). ESBL-producing Enterobacteriaceae were detected in five samples of human sewage in Spain (Mesa *et al*., 2006).

The human colon is the major reservoir of emerging opportunistic pathogens such as *E. coli, Klebsiella*, *Enterobacter*, and *Acinetobacter baumanii* (Agustía *et al*., 2002; Fanaro *et al*., 2003; Hollander *et al*., 2001) and it is likely that these are food-derived in the community (Turtura *et al*., 1990). The distinction between food-borne commensals, pathogens and nosocomial pathogens is some what arbitrary, and many emerging nosocomial Gram negative pathogens may be food borne, normally living in the gut as commensals until the individual becomes immunosuppressed or until antibiotic resistance genes transferred from another organism. Recent research has revealed that soil, particularly the plant rhizosphere, harbors diverse opportunistic human pathogens, including *Acinetobacter baumanii, Aeromonas salmonicida, Burkholderia cepacia, Enterobacter agglomorans, Klebsiella pneumonia, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella typhimurium, Serratia* sp., *Staphylococcus aureus, Stenotrophomonas maltophilia*, and others (Berg *et al*., 2005).

B. Farm animals

When soils are treated with manure both the residues of antibiotics used as veterinary medicines and the bacteria carrying genes conferring resistance are introduced into the soil and reach the food chain, for example, via plant-associated bacteria (Witte *et al*., 2000). Some 461 tonnes (active ingredient) of antimicrobial therapeutics and growth promoters were sold for use in food animals in 2000 in the UK (National Office of Animal Health data), including tetracyclines (228 t), trimethoprim/sulphonamides (94 t), β-lactams (49 t), macrolides (41 t), and FQs (1 t). Some veterinary antibiotics are synthetic, so unlike those produced by soil bacteria, many cannot be broken down through normal processes, and therefore may persist for a long time in soils; adsorption to soil particles and other surfaces allows accumulation of residual antibiotics to high concentrations (Kummerer, 2004).

It has been clearly established that the use of certain antibiotics in agriculture has contributed to the development of resistant bacterial strains in human infection, as evidenced by the work of Wolfgang Witte and others where vancomycin resistance genes in human *Enterococcus faecalis* isolates were traced to the use of avoparcin in pigs (Witte, 1997). As early as the 1970s Stuart Levy noted that oxytetracycline was a major

feed additive and that studies had shown there was a strong association between tetracycline resistance in isolates from livestock and animal workers (Levy *et al*., 1976). In 1969 the Swann committee report recommended that antibiotics used in human medicine should not be used as growth promoters. Only in the last few years have a number of antibiotics been banned, and clearly their use as growth promoters is inadvisable.

A mixture of the parent product and metabolites are excreted in faeces and urine. Excreta enter the farm environment directly in grazing animals and indirectly in intensively reared animals through application of slurry and manure. It is estimated that approximately 70 million tons of animal manure wastes are spread onto agricultural land per annum in the UK (Hutchison *et al*., 2004). In many river catchments the bulk of faecal coliforms are believed to be of agricultural origin. Chee-Sanford *et al.* (2001) screened for eight *tet* genes in groundwater associated with swine production facilities. Tetracycline resistance genes were found as far as 250 m downstream from waste lagoons, highlighting the danger posed by use of antibiotics in agriculture and the risk of contamination of drinking water with antibiotic resistant bacteria. In a different study a detection limit of 10^2-10^3 copies of the *tet*(M) gene per gram was achieved using a nested PCR method with TC-DNA (Agerso *et al*., 2004). The gene was detected in farmland soil previously amended with pig slurry containing resistant bacteria; the number of positive samples from farmland soils one year after manure treatment was significantly higher than in samples of garden soil not treated with manure.

E. coli strains producing an ESBL (CTX-M-2) were recently isolated from cattle faeces in Japan (Shiraki *et al*., 2004). β-lactamases and ESBLs were also detected in *E. coli* isolates from healthy chickens, food and sick animals in Spain (Brinas *et al*., 2002, 2003a,b). The use of extended-spectrum cephalosporins in chickens is very unusual, and the possibility of cross-selection with other antimicrobials used in poultry (such as sulphonamides and tetracyclines, etc.) might explain this discovery. In a study of retail chicken breasts, quinolone resistant *E. coli* also producing CTX-M-2 were found in 5 of 10 samples produced in Brazil (CTX-M-2 is widely reported in human infection with *Salmonella* and *E. coli* from South America), whereas only 1 of 62 samples of UK produced chicken were positive for CTX-M-1 (Ensor *et al.*, 2007). ESBLs were also discovered in samples from 8 of 10 pig farms, 2 of 10 rabbit farms, from all 10 poultry farms tested and in 3 of 738 food samples studied in Spain (Mesa *et al*., 2006).

The FQ antibiotic enrofloxacin (the major metabolite being ciprofloxacin) is used extensively in poultry farming and evolution of fluoroquniolone resistance in chicken litter was documented, caused by mutation in the QRDR of the *gyrA* gene (Lee *et al*., 2005). Plasmid-mediated quinolone resistance (PMQR) is also known to occur. The gene responsible for PMQR was identified as *qnr* and this gene was found in an integron-like

element and is associated with the ESBL VEB-1 (Poirel *et al*., 2005c). The origin of *qnrA* was recently established as the water-borne species *Shewanella algae*, which, in addition to the fact that CTX-M-ases originate in the rhizosphere bacteria *Kluyvera* sp., underlines the role of the environment itself as a reservoir of novel resistance genes (Nordmann and Poirel, 2005; Poirel *et al*., 2005b). Recently, further transferable quinolone resistance genes *qnrB* and *qnrS* have been identified in Gram negative opportunistic pathogens (Poirel *et al*., 2005a). In 2004, an entirely novel plasmid mediated mechanism of quinolone resistance was discovered. In *E. coli* from Shanghai, PRC strains with a MIC of 1.0 mg/liter of ciprofloxacin carried a mutated form of the aminoglycoside inactivating enzyme AAC (6^1)-Ib-cr (Robicsek *et al*., 2006). This enzyme N-acetylates quinolones that have an amino nitrogen on the piperazinyl substituent (e.g., ciprofloxacin and norfloxacin). The distribution of the gene has not been studied extensively but has been found in China and North America (Robicsek *et al*., 2006). It was recently reported to be associated with the ESBL genes $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1}}$, and $bla_{\text{TEM-1}}$ on a limited range of plasmids carried by *E. coli* in Portugal (Machado *et al*., 2006). The consequences could be grave as ciprofloxacin is extensively used in agriculture and is the principal metabolite of veterinary quinolones such as enrofloxacin. The use of quinolones (which are also slow to degrade in the environment) will coselect for a wide range of *qnr/acc* (6^1) associated resistances such as resistance to 3rd generation cephalosporins and carbapenems. Thus, there is increasing evidence of an environmental reservoir of clinically important antibiotic resistance genes.

Several studies demonstrated a correlation between the extent of use of antibiotics in animals and the incidence of the respective antibiotic resistance genes. Therefore it is not surprising to learn that animal manure, in particular piggery manure, has been shown to be a ''hot spot'' (high incidence) of bacteria carrying antibiotic resistance genes residing on mobile genetic elements (Smalla *et al*., 2000).

Antibiotic resistance does not always appear to be directly related to short-term trends in antibiotic usage. The glycopeptide growth promoter avoparcin was banned from animal production in Denmark in 1995, and in the EU in 1997, because of concern for the spread of vancomycin-resistant enterococci (VRE) from food animals to humans. A Danish study found high levels of VRE in broiler flocks five years after avoparcin was withdrawn (Heuer *et al*., 2002a). Further studies revealed that VRE was surviving in broiler houses despite cleaning procedures between production rotations (Heuer *et al*., 2002b).

C. Transfer from the environment to the clinic

Patients admitted to hospital are likely to acquire bacteria that are multiplying in the hospital environment, such as *Pseudomonas aeruginosa* and these may well be antimicrobial resistant. It has been demonstrated that the general environment of the hospital, particularly sites such as sink drains, mop heads, and other wet environments, will act as sources not only of bacteria capable of directly causing nosocomial infection but which can also act as a gene pool of antibiotic resistance genes. Apart from this obvious interaction of patients with the hospital environment, it is probably food that is the major route of flow of resistance genes from the more general environment to man. The human gut carries a large number of commensal bacteria, usually in the order of $\geq 10^{10}$/g of faeces and this value applies only to culturable bacteria. The bowel flora is constantly being challenged by new bacteria in food and although the dominant flora remains, colonization with a minority of antibiotic resistant bacteria, particularly Enterobacteriaceae/enterococci and staphylococci, occurs in those individuals not receiving antimicrobials. The size and complexity of this bowel flora reservoir, which is in direct connection with the environment, has long been recognized as containing large numbers of antibiotic resistant bacteria. A study undertaken as early as 1979 showed that in individuals with no history of recent consumption of antibiotics, 10% or more of the total aerobic Gram negative bacteria were resistant to one or more antimicrobials (Levy *et al*., 1988). This pool of resistance genes may get transferred into bacteria with significant pathogenicity towards humans, or in very many cases, opportunistic pathogenic bacteria such as *E. coli, Klebsiella* sp., entrococci, and *Staphylococcus aureus*, which may cause infections in the individual. In this way, there is a continuous link between selection for antibiotic resistance in the general and agricultural environment and human medicine. The other major commensal flora site on humans is the skin surface and recently with the recognition of community acquired MRSA, the importance of antibiotic resistant staphylococci and other Gram positive bacteria on the skin, and the transfer into potentially pathogenic species such as *Staphylococcus aureus* has been emphasized. As with the large bowel, all the studies clearly show that even those individuals not receiving antibiotics carry a substantial load of antibiotic resistant bacteria, which must be strongly influenced by interactions with other humans, companion and food animals. An extensive study by Cove and colleagues showed that the incidence of seven primary antibiotic resistance markers among the staphylococcal flora in antibiotic untreated subjects was tetracycline 87.5%, erythromycin 68.8%, fusidic acid 56.3%, trimethoprim 42.4%, chloramphenicol 25%, clindamycin 9.4%, and gentamicin 4.7%. We now recognize that among staphylococci there is ample opportunity and genetic

mechanisms for the mobilization of resistance genes into potentially pathogenic species (Cove *et al*., 1990). Some special groups in the human community such as farm workers, medical personnel and patients receiving antimicrobials, have a much higher incidence of colonization with antibiotic resistant bacteria, some of which, such as in the case of farm workers, are derived from contact with farm animals that have either been treated with antimicrobials or exposed to selecting agents. Recent problems with community acquired MRSA in pig farmers illustrate the way in which problems can arise rapidly, and undermine the use of clinically important antimicrobials.

V. MRSA IN THE NONCLINICAL ENVIRONMENT

A. Methicillin resistance in *Staphylococcus aureus*

Staphylococcus aureus is well known for its ability to acquire antibiotic resistance, both historically in relation to penicillin, erythromycin, and tetracycline and more recently methicillin and vancomycin resistance. The acronym MRSA (Methicillin resistant *S. aureus*) is feared by healthcare professionals the world over. *S. aureus* forms part of the normal human flora, residing asymptomatically in the mucosal linings of healthy individuals and at other moist skin sites (Hiramatsu *et al*., 2001; Peacock *et al*., 2001) and is particularly pathogenic in individuals at the extremes of age who have intravascular/urinary catheters, diabetes, and other compromising coexisting medical conditions (Lindsay and Holden, 2004). However, recent years have seen a rise in highly virulent community acquired strains (CA-MRSA) capable of causing disease in young, healthy individuals with none of the prescribed risk factors. Resistance to methicillin is carried by SCC*mec*, a mobile genetic island that can disseminate horizontally, although its mode of transfer is currently unknown (Hanssen and Ericson Sollid, 2006). Resistance is encoded by the *mec*A gene (Ito *et al*., 1999), *mec*A encodes an attenuated penicillin binding protein (PBP2′ or PBP2a), which has a lower affinity for penicillin and other β-lactams than the innate PBPs (Hartman and Tomasz, 1981), hence interfering with antimicrobial activity. There are six basic described SCC*mec* types; additional resistance genes may be present or absent depending on the type. SCC*mec* is inserted into the *S. aureus* chromosome near the origin of replication, always at the same location at the 3′ end of the *orf*X gene (Kuroda *et al*., 2001). Its origins are unknown, but as no methicillin-susceptible *S. aureus* (MSSA) homolog exists (Archer and Niemeyer, 1994) it has been suggested that it was transferred horizontally from a coagulase-negative staphylococcus (CoNS) such as *S. sciuri* (Couto *et al*., 1996, 2003). The conjugative transposon containing *tet*M

(tetracycline resistance) has been suggested to pass between *Clostridia* and staphylococci (Ito *et al*., 2003), and empirical support for the movement of mobile elements between staphylococci and other low GC Gram negative bacteria (Gill *et al*., 2005), and the potential transfer of *van*A (vancomycin resistance) from *Enterococcus faecalis* to *S. aureus* (Weigel *et al*., 2003) lends weight to the possibility of a common gene pool available to many bacterial species (Hanssen *et al*., 2004). Movement of DNA into *S. aureus* is tightly controlled by a restriction-modification system Sau1, a type 1 system that has been found on the chromosome of all known sequenced strains (Waldron and Lindsay, 2006).

Although much of the genome of *S. aureus* consists of mobile genetic elements, because of the presence of Sau1, horizontal transfer is likely to be more frequent among members of the same lineage, as the Sau1 restriction-modification system present in different lineages have specific differences (Waldron and Lindsay, 2006). This is thought to explain the rare occurrence of *vanA* carrying VRSA and the limited number of lineages with SCC*mec* (CC1, CC5, CC8, CC22, CC30 and CC45). Possession of SCC*mec* is thought to carry a fitness cost and will therefore be selected for only when strains have been exposed to antibiotics (Katayama *et al*., 2003).

B. Environmental reservoirs of MRSA

Although antibiotic selective pressure present in clinical environments is clearly a major source of MRSA infection (Dar *et al*., 2006), environmental reservoirs have been implicated in the spread of resistant strains. Subinhibitory levels of antibiotics may be to blame for inducing resistance in commensal bacteria in farm animals, inducing resistance in pathogenic bacteria through plasmid transfer (Singer *et al*., 2003). In pig and poultry farming, heavy use of antibiotics in typically intensively farmed settings predisposes them to MRSA colonization (Shea, 2004; van Den Bogaard *et al*., 2000). Close contact between animals is inevitable, and infections are likely to spread quickly. Bacterial transmission in humans is (or should be) easily negated through regular hand washing, whereas oral-faecal contact cannot be prevented in animals and fast transmission of faecal-borne disease is unavoidable (van den Bogaard and Stobberingh, 1999). Therefore, an infection in one or two animals is often combated by a blanket treatment with antibiotics of the entire house, which may contain over 10,000 animals (Shea, 2004). The farming practice employed may have some bearing on the antibiotic susceptibility of commensal *S. aureus*; organic farming may result in fewer resistant bacteria than in conventional farms because of lower exposure to antibiotics and reduced contact between animals (Halbert *et al*., 2006; Sato *et al*., 2005; Tikofsky *et al*., 2003). There is some contrary evidence (Sato *et al*., 2004), but the continued use

of antibiotics in some of the organic farms studied was a possibility (Busato *et al*., 2000).

Cross-resistance for methicillin and some cephalosporins has been demonstrated (Hansen-Nord *et al*., 1988; Menzies *et al*., 1987), with administration of cephalosporins increasing the acquisition of nosocomial MRSA three-fold (Asensio *et al*., 1996). Similarly, tetracycline resistance may be caused by one of several genes, one of which, *tet*K, is carried by a plasmid (pT181) that is inserted into SCC*mec*III (Ito *et al*., 2003). As tetracycline concentrations have been found to persist at high concentrations long after slurry application has ceased (De Liguoro *et al*., 2003; Hamscher *et al*., 2002), selection for tetracycline resistance may contribute to methicillin resistance as a result of selection for SCC*mec*III; although SCC*mec*IV is far more common in community MRSA. In the absence of antibiotics, the presence of quaternary ammonium compounds (QACs) in soils, used extensively as disinfectants, may also coselect for β-lactam resistance (Sidhu *et al*., 2001, 2002); resistance to QACs correlates with the β-lactamase transposon Tn552 because of co-carriage of the *bla*Z gene and *qac*A (Anthonisen *et al*., 2002). Evidently, the introduction of any of these compounds to the environment may lead to selection for methicillin resistance in *S. aureus* colonizing or infecting farm animals, or in other bacteria inhabiting the soil; there is some evidence to suggest colonization of the rhizosphere by *S. aureus* may be possible, so acquisition of methicillin resistance may occur both in soil and in farm animals (Berg *et al*., 2005; Germida and Siciliano, 2001; Morales *et al*., 1996).

C. Pig associated MRSA

Antibiotic resistance levels vary from country to country. For example, the percentage of MRSA in the UK (40% of nosocomial *S. aureus* isolates causing bacteremia) contrasts sharply with that in Holland, where it is currently very rare, making up only 1% of *S. aureus* isolates (Tiemersma *et al*., 2004). In Holland most cases are found in people who have recently attended foreign medical institutions; however, occasional instances have arisen, whereby foreign travel was not a contributing factor. One such case described an MRSA positive baby that had never travelled abroad (Voss *et al*., 2005). The family lived on a pig farm, and MRSA was isolated from one of the pigs. Two further cases were reported in the same study, both of which were associated in some way with pig farming. As pig farming was the only common denominator in these cases it was deduced that there may be a link between pig farming and increased risk of MRSA infection; indeed colonization by MRSA in pig farmers was found to be almost twice that of the general population (Aubry-Damon *et al*., 2004). Pigs have been similarly implicated as a reservoir of MRSA in France, a study of pig isolates found all to be nontypeable by pulsed-field gel

electrophoresis (PFGE), the so-called gold standard for MRSA typing (Armand-Lefevre *et al*., 2005), and the association between PFGE nontypeable strains and pig farming has gained further empirical support (Huijsdens *et al*., 2006). The three multilocus sequence types (MLSTs) ST9, ST398, and ST433 were found in pigs and only in humans associated with pig farming suggesting an association between these STs and pigs (Armand-Lefevre *et al*., 2005). These findings were supported by a recent Dutch study in which 39% of all pigs sampled from slaughter houses (540 pigs) carried MRSA in their nares, and all of these belonged to ST398 (de Neeling *et al*., 2007). Obviously Holland represents an unusual case because of its low MRSA incidence, but the high percentage of swine carriers, all with the same MLST type, suggests a recent dissemination of MRSA. The vast majority of isolates contained SCC*mec* types IV and V, indicating a relatively frequent transfer of this mobile element; types IV and V are suspected to transfer at a greater rate than the other types (Daum *et al*., 2002). As pig-associated strains seem to be passed from human to human as well as between humans and pigs, pig farming may represent a source of CA-MRSA (Huijsdens *et al*., 2006). CA-MRSA is susceptible to a greater range of antibiotics than its hospital-acquired counterpart (Herold *et al*., 1998), but tends to be more virulent because of the acquisition of toxin genes such as the Panton-Valentine Leukocidin (PVL) (Boyle-Vavra and Daum, 2007). PVL has not yet been found in pig-associated strains (Huijsdens *et al*., 2006), but more studies need to be conducted to confirm this. There are relatively few CA-MRSA strains, with six currently recognized (Eady and Cove, 2003). It has been suggested that these strains are associated with particular niches (Shukla, 2005), concurring with the potential association of the already mentioned sequence types with pigs and pig farmers. The antibiotic resistance patterns of pig MRSA, other than the resistance to methicillin, reflect the antibiotics used in veterinary medicine: tetracycline resistance is very common, whereas ciprofloxacin resistance has been found to be completely absent (de Neeling *et al*., 2007). This is very different from the situation in nosocomial MRSA strains, in which over 80% of MRSAs may be resistant to ciprofloxacin (Marangon *et al*., 2004; Raviglione *et al*., 1990), perhaps suggesting that ST398 is circulating in pigs and only infecting humans occasionally, or that pigs are an emerging reservoir of MRSA.

D. Cattle associated MRSA

MRSA is comparatively rare in cows, and those *S. aureus* found to be resistant to methicillin tend to lack SCC*mec*, gaining resistance from the production of β-lactamases (De Oliveira *et al*., 2000). Penicillin and ampicillin resistance are more widespread (Gentilini *et al*., 2000; Guler *et al*.,

2005) because of the frequent use of these antibiotics in treating intramammary infections (Pengov and Ceru, 2003). Antibacterial treatment for mastitis has been implicated as a major cause of resistance in bacterial isolates from treated animals, other animals within the herd, and in meat products intended for human consumption (Berghash *et al*., 1983; Chen *et al*., 2004; Griggs *et al*., 1994; Piddock, 1996). Furthermore, the secretion of antibiotics in milk produced by cows under treatment for mastitis is one of the most common causes of illegal antibacterial residues in milk (Erskine, 1996). Although cattle have a greater freedom of movement than intensively farmed pigs and poultry, effectively reducing the risk of transmission, other risk factors are present. There are extreme inconsistencies in the therapeutic success rates of the standard treatment regimes (Dingwell *et al*., 2003), with chronic infections being cured through intramammary antibiotic infusions only 35% of the time (Owens *et al*., 1997). Indiscriminate use of antibiotics, incomplete treatments, and incorrect diagnoses of antibiotic resistant strains do not help the situation (Turutoglu *et al*., 2006). Although methicillin is not generally used in treatment of cows, MRSA is increasing in its significance as a cause of bovine mastitis (Bernabé *et al*., 2005; Turutoglu *et al*., 2006). Acquisition of MRSA may occur through direct contact with humans: one outbreak of mastitis has been attributed to contact with a farm worker (Devriese *et al*., 1986), and more recent empirical evidence concurs with this (Fox *et al*., 1991; Roberson *et al*., 1994). However, this is slightly contentious as evidence to the contrary suggests that transmission between humans and cows is rare (Kapur *et al*., 1995; Lopes *et al*., 1990) because of the host specificity of *S. aureus* clones (Smith *et al*., 2005).

Environmental survival of MRSA may contribute to bovine infections, as shown in the clinical setting as discussed by Hardy *et al*. (2004), where it may survive for several months and act as a source for infecting patients (Neely and Maley, 2000; Sasatsu *et al*., 1993). Biofilm formation on various materials allows persistence and avoidance of desiccation (Gotz, 2002), so there is a risk of transmission from cow to cow via milking devices and other surfaces. Despite the move towards automated milking practices, reducing contact between humans and untreated milk, the production of aerosols in milking parlors may put workers, as well as other cows, at risk from contamination (Roberson *et al*., 1994). Similarly, aerial contamination has been noted in pig barns (Gibbs *et al*., 2004), and in aerosols produced by confined swine-feeding operations (Chapin *et al*., 2005; Sapkota *et al*., 2006). Pig feeds have been implicated as potential sources of contamination of tetracycline-resistant *S. aureus*, which may thrive in tetracycline enriched feeds (de Neeling *et al*., 2007).

E. Horse associated MRSA

MRSA has also been identified as an emerging disease in horses (Weese *et al*., 2005a,b, 2006), which might act as a reservoir of rare strains for transmission to humans (Baptiste *et al*., 2005). The isolation of MRSA from two horses in Canada led to a larger study, which revealed the colonization of 79 hospitalized horses and also of 27 people involved in caring for these animals, strongly suggestive of frequent transmission between horses and humans (Weese *et al*., 2005b). All isolates in the study were from nasal swabs and belonged to the Canadian community associated strain C-MRSA5, which is rare in humans. Thus horses may act as a reservoir for this strain, perhaps mirroring the putative association of ST398 with pigs. In Slovenia, MRSA was absent from a total of 300 horses sampled with nasal swabs (Vengust *et al*., 2006); one explanation for this is that MRSA may also colonize the perineum or the throat, and this has been found to be the sole site of infection in some cases (Coello *et al*., 1994), so it is possible that subclinical carriers of MRSA were missed. Indeed, a study on humans in nursing homes indicated that up to 14% of cases were missed by screening the nares alone (Lee *et al*., 1997). MRSA colonization tends to be transient in nature, and so may be missed on screening, whereas it has been shown that infections are likely to persist in hospitalized horses, particularly in those that have undergone invasive procedures (Seguin *et al*., 1999).

F. MRSA in companion animals

The situation in companion animals differs from that seen in pigs, as there does not appear to be any association with a particular MRSA clone. The same strains are found in humans and companion animals (van Duijkeren *et al*., 2003), indicating that pets acquire the infection from humans. Although *S. intermedius* is the predominant staphylococcal species in dogs (Biberstein *et al*., 1984; Hoekstra and Paulton, 2002), *S. aureus* is also frequently found on the skin or associated with suppurative infections (Kloos, 1990). Dogs may act as a reservoir of MRSA (Baptiste *et al*., 2005) and have been implicated in causing unexplained relapses in humans previously treated and cleared of MRSA infection (Manian, 2003; van Duijkeren *et al*., 2003). Strains isolated from humans and dogs have been found to have the same PFGE patterns (Manian, 2003) and SCC*mec* types (van Duijkeren *et al*., 2004). Following treatment of dogs, relapses of infection in the owners ceased (Cefai *et al*., 1994; Manian, 2003; van Duijkeren *et al*., 2004). Although, like dogs, cats may also carry MRSA, *S. aureus* is not the most common staphylococcal infection; in cats this is fulfilled by a combination of *S. intermedius* and the CoNS *S. felis* (Lilenbaum *et al*., 1998; Patel *et al*., 1999). Surgery is a risk factor in cats and dogs just as it is

in humans, making up the vast majority of MRSA cases (Boag *et al*., 2004; Owen *et al*., 2004; Tomlin *et al*., 1999; van Duijkeren *et al*., 2003).

The epidemiology of MRSA appears to be changing, with the possibility of animals acting as reservoirs of infection for people without any of the usual risk factors. In countries that currently have a low incidence of MRSA, this may be an important source of an otherwise rare infection.

VI. CONCLUSIONS

The evolution of antibiotic resistant bacteria is one of the most significant problems in modern medicine and poses a serious threat to human health. Increasingly the huge diversity of resistance genes that already exist in the environment is beginning to be appreciated. Understanding the selective pressures and mechanisms of gene transfer that drive dissemination of resistance genes not only in the clinic, but also in the wider environment is crucial for long-term strategies in the treatment of microbial disease.

Modern farming practice is attempting to reduce dependency on antibiotics but this in itself may not reduce particular mechanisms of resistance such as genes carried on class 1 integrons, which we have shown can be selected for by biocides in the environment. Understanding the ecology of resistance genes is extremely difficult as genes may be carried by unculturable bacteria (99.0–99.9% of bacteria). Movement of genes between environmental bacteria and the clinic has therefore been difficult to investigate in the past. However, modern molecular approaches such as epidemiological studies of key resistance determinants in total community DNA using quantitative real-time PCR allows detailed analyses and comparison of gene prevalence in the environment and human gut. Functional metagenomics and integron clone library construction allows the entire resistance gene pool or metagenome to be analyzed. Antibiotic resistant bacteria in the environment may be transferred to the human population via ingestion of contaminated food and water, via direct contact with animals, swimming in lakes, rivers, and the sea, and by airborne bacteria. In reality, there is no distinction between clinical and nonclinical environments; both must be considered to fully understand the underlying causes of antibiotic resistance in clinically important bacteria.

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CHAPTER 8

Host Defense Peptides in the Oral Cavity

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I. INTRODUCTION

Since their discovery more than 30 years ago within phagocytic granules and as components of the innate defenses of insects and amphibians, antimicrobial peptides have emerged as important multifunctional components of the innate defenses of higher and lower organisms (Boman, 1995; Lehrer, 2004; Zasloff, 2002). These structurally diverse, small (up to 50–100 amino acids in length), cationic peptides display varying antimicrobial activity against bacteria, viruses, and fungi. They also have key activities in regulating innate and adaptive responses and in suppressing host responses to inflammatory products, especially lipopolysaccharide (LPS) (Scott *et al*., 2002; Yang and Oppenheim, 2004). This multiplicity of function, and the fact that at physiological concentrations many peptides would display immunomodulatory but not antimicrobial effects, led recently to the suggestion that they should be termed ''host defense peptides'' (HDPs) to more accurately reflect their roles in nature (Hancock and Devine, 2004).

HDPs have additional importance as novel therapeutic anti-infective, anti-sepsis, anti-cancer and anti-inflammatory molecules (Hancock and Sahl, 2006). We are experiencing a period of significant change with respect to the use of conventional antimicrobial therapy to combat disease. With the threat of widespread antibiotic resistance rendering many antibiotics useless against significant infections, there is an increased necessity to minimize antibiotic use and an impetus to develop nontraditional antimicrobial or immunomodulatory agents based on natural products such as HDPs, as well as to raise the profile of disease prevention.

Oral diseases are among the most common human infectious diseases and they have significant impacts on quality of life and general well-being, as well as imposing a huge economic cost worldwide (Petersen *et al*., 2005). Many common oral diseases arise from the activities of, and host responses to, consortia of microorganisms that are selected from the normal resident populations following disruption of microbial ecology or host–microbe homeostasis. The resident populations of the mouth comprise large numbers of diverse organisms, which grow within biofilm communities attached to hard and soft tissues; oral biofilms are the best characterized complex biofilms of relevance to human health and disease (Kolenbrander *et al*., 2006; Marsh, 2005). HDPs expressed by tissues throughout the mouth have potential significance in oral host–microbe homeostasis, in defense against oral pathogens, predisposition to disease, and natural and synthetic peptides are being developed as novel therapeutic agents for treatment of oral diseases. To fully assess their significance and elucidate their roles in health and disease it is essential to understand host–microbe interactions in the mouth, where HDPs interact with a huge diversity of organisms.

II. HOST–MICROBE INTERACTIONS IN THE MOUTH

More than 700 bacterial species have been isolated from the human mouth (Paster *et al*., 2006), and only 50–60% of these organisms can currently be cultured. The reason for this, in part, is probably that they have evolved to live within a biofilm community of interdependent species and will not grow in monoculture (Handelsman, 2004; Wade, 2002). Within biofilms, resident bacteria gain significant advantages, that is, protection from host defenses and antimicrobial agents; expression of resistant phenotypes, interspecies communication, and horizontal gene transfer; and the development of food-webs and interactions (Marsh, 2005; Roberts and Mullany, 2006). Thus, oral HDPs interact with microorganisms that are not only extremely diverse, but that are also protected within (and adapted to) a sessile, community-based mode of living.

A. The normal oral microbiota

Resident commensal populations protect tissues from colonization by exogenous pathogens, promote normal development of host cell structure and function, ensure normal development of the immune system, and damp-down immune responses (Devine, 2004). Existing paradigms concerning host–commensal interactions have arisen mainly from studies of responses to gut bacteria. Preliminary studies have indicated a comparable beneficial role for oral commensal bacteria, in that they determine normal expression of immune mediators (Dixon *et al*., 2004b), can suppress epithelial cell cytokine responses (Cosseau *et al*., submitted) (Hasegawa *et al*., 2007), and provide protection against colonization by exogenous organisms (Marsh, 2005).

The mouth is unique in possessing large, nonshedding, hard tissue surfaces (teeth) as well as a variety of mucosal surfaces that become colonized by distinct resident microbial communities. These communities exhibit considerable species- and genetic-diversity (Kilian *et al*., 2006), which is further increased through their rapid adaptation to changes in environmental conditions that vary between tissues, cells, and serum, and following inflammation and bleeding. Colonization of certain sites provides reservoirs that facilitate dispersal of organisms to other sites, for example, organisms on the dorsum of the tongue are reservoirs for supragingival and subgingival plaque and salivary populations (Beighton *et al*., 1987; Gibbons, 1989; Mager *et al*., 2003). Many oral bacteria survive within buccal epithelial cells, the frequent desquamation of which may represent another mechanism for dispersal (Rudney *et al*., 2005a,b). The composition of resident populations is highly site-specific (Aas *et al*., 2005; Mager *et al*., 2003; Paster *et al*., 2006) because of physicochemical differences

between sites, the specificity of adhesive interactions that occur between microbe and host, as well as the activities of the host defenses.

The pioneer species that colonize teeth are mainly streptococci, particularly *Streptococcus mitis* and *Streptococcus oralis*, with smaller proportions of *Actinomyces* and other species. Sequential and specific adhesive interactions and bacterial successions result in a complex climax supragingival plaque community above the gingival-tooth margin (Kolenbrander *et al*., 2006). Subgingival plaque is derived from supragingival plaque that spreads down into the gingival sulcus (Aas *et al*., 2005; Kolenbrander *et al*., 2006). Certain consortia of organisms are commonly isolated from healthy but not diseased sites (Socransky and Haffajee, 2005). *S. mitis, S. oralis, Actinomyces naeslundii, Fusobacterium nucleatum, Haemophilus parainfluenzae, Eikenella corrodens,* and some species of *Prevotella* have been proposed to be ''true'' oral commensals (Kilian *et al*., 2006). *F. nucleatum* has a particularly important role in plaque maturation, as it is promiscuous in its coaggregation partners and is a bridging organism between early and late colonizers (Kolenbrander *et al*., 2006).

B. Microbiota associated with disease

The major common microbial diseases that occur in the mouth are polymicrobial infections caused by the actions of organisms that arise from the normal microbiota through processes of selection and succession. In dental caries (tooth decay), dietary intake of fermentable carbohydrates facilitates the selection from within supragingival plaque of consortia of aciduric (acid tolerant) and acidogenic bacteria, in particular streptococci and lactobacilli, while the production of acid results in the dissolution of enamel and lesion formation (Marsh, 2003). The organisms implicated in the etiology of caries include the mutans streptococci, particularly *Streptococcus mutans* and *Streptococcus sobrinus*, lactobacilli, and other acid tolerating streptococci (Babaahmady *et al*., 1997; Boyar and Bowden, 1985; Tanner *et al*., 2002). Caries progression and deepening of lesions can allow bacterial access into dentinal tubules and pulp, causing pulpitis and pulp necrosis (Love and Jenkinson, 2002).

Periodontal diseases (gingivitis and periodontitis) are common inflammatory conditions that occur in response to subgingival plaque biofilms and severe disease is also associated with other significant conditions such as diabetes (Loe, 1993) and HIV infection (Robinson, 1997). Gingivitis is a reversible inflammation, while periodontitis involves gingival inflammation with apical migration of epithelial attachment to the root surface and accompanying loss of periodontal ligament and alveolar bone (Armitage, 1999). The stagnant periodontal pocket that is formed may harbor up to 10^8 diverse bacteria (Kilian *et al*., 2006; Paster *et al*., 2006; Socransky *et al*., 1991). Disease occurs following a transition from the

predominantly Gram-positive facultative populations associated with health to plaque that is dominated by obligately anaerobic, proteolytic Gram-negative rods and spirochetes, many of which are unculturable (Ellen and Galimanas, 2005; Kolenbrander *et al*., 2006). Tissue damage and disease progression occur as a result of the combined activities of organisms within subgingival biofilms and, paradoxically, host responses to them (Dixon *et al*., 2004a; Kirkwood *et al*., 2007). These result in an exaggerated inflammatory response, and much of the tissue damage observed in periodontitis is mediated by inflammatory host factors. Inflammatory processes also provide environmental and ecological stimuli (e.g., increased gingival crevicular fluid flow, increased pH, lower redox potential, increased availability of peptide nutrients, and heme sources) that drive bacterial successions within subgingival plaque and the emergence of populations associated with disease (Marsh, 2003).

Periodontal diseases are complex polymicrobial conditions and many organisms have been implicated in their etiology (some of which are listed in Table 8.1). *Aggregatibacterium actinomycetemcomitans* (previously *Actinobacillus actinomycetemcomitans)* (Norskov-Lauritsen and Kilian, 2006) is strongly associated with aggressive localized forms of periodontitis (Fine *et al*., 2006). Socransky *et al.* identified six complexes of culturable bacteria with different associations with health and disease (Socransky and Haffajee, 2005) including the ''Red complex'' that is most strongly associated with severe chronic periodontitis (Table 8.1). The 12 organisms in the Orange complex (Table 8.1) form a consortium, whose colonization is thought to be essential for the subsequent succession of the Red complex organisms. Other groups have found additional culturable and nonculturable organisms to be significant in periodontitis (Brinig *et al*., 2003; Dahlen and Leonhardt, 2006; Kumar *et al*., 2003; Lepp *et al*., 2004; Teles *et al*., 2006). Notwithstanding their association with disease, many putative periodontal pathogens are often detected (albeit in low numbers) in periodontally healthy individuals or at healthy sites, as well as in the plaque of young children and adolescents, and *P. gingivalis* and *A. actinomycetemcomitans* resemble commensals in their genetic diversity, acquisition, and population structures (Kilian *et al*., 2006). With the exception of the *A. actinomycetemcomitans* JP2 clone, they function as opportunistic pathogens, and clones of each species vary in their pathogenicity.

Candida albicans is present in low levels in resident oral populations and it causes denture-associated stomatitis through its ability to colonize polymethyl methacrylate materials. It is also a significant opportunistic pathogen, and oral candidiasis is a common infection of people who are immunocompromised or have undergone radiotherapy or antibiotic therapy (Ramage *et al*., 2004; Redding *et al*., 2004).

Herpes simplex virus (HSV) types 1 and 2, and other herpesviruses, are commonly isolated from oral tissues and secretions, as are papilloma

TABLE 8.1 Subgingival microorganisms associated with periodontitis

Organism	Association with disease	Reference
CULTURABLE BACTERIA		
Porphyromonas gingivalis[a]	Severe chronic periodontitis	Socransky and Haffajee, 2005
Tannerella forsythia[a]	Severe chronic periodontitis	Socransky and Haffajee, 2005
Treponema denticola[a]	Severe chronic periodontitis	Socransky and Haffajee, 2005
Aggregatibacterium actinomycetemcomitans	Aggressive localized forms of periodontitis	Fine *et al*., 2006
A. actinomycetemcomitans JP2 clone	Aggressive periodontal disease in people of North African decent	Kilian *et al*., 2006
Prevotella intermedia[b]	Early/moderate periodontitis	Socransky and Haffajee, 2005
Prevotella nigrescens[b]	Early/moderate periodontitis	Socransky and Haffajee, 2005
Micromonas micros[b]	Early/moderate periodontitis	Socransky and Haffajee, 2005
Fusobacterium nucleatum 3 subspecies[b]	Early/moderate periodontitis	Socransky and Haffajee, 2005
Campylobacter gracilis[b]	Early/moderate periodontitis	Socransky and Haffajee, 2005
Campylobacter rectus[b]	Early/moderate periodontitis	Socransky and Haffajee, 2005
Campylobacter showae[b]	Early/moderate periodontitis	Socransky and Haffajee, 2005
Selenomonas noxia	Early/moderate periodontitis	Tanner *et al*., 1998
Prevotella tannerae	Periodontitis	Dahlen and Leonhardt, 2006

Filifactor alocis	Periodontitis	Dahlen and Leonhardt, 2006
Porphyromonas endodontalis	Periodontitis	Dahlen and Leonhardt, 2006
Dialister pneumosintes	Periodontitis	Teles *et al*., 2006
Streptococcus milleri group	Periodontitis	Teles *et al*., 2006
Treponema socranskii	Periodontitis	Teles *et al*., 2006
UNCULTURABLE BACTERIA		
Spirochetes	Periodontitis	Ellen and Galimanas, 2005
TM7 phylum	Early periodontitis	Brinig *et al*., 2003
Methanogens	Severe periodontitis	Lepp *et al*., 2004
6 Novel phylotypes	Periodontitis	Kumar *et al*., 2003

[a] Organisms of the ''Red Complex''; one of six groups of culturable bacteria with different associations with health and disease, and the consortium most strongly associated with severe chronic periodontitis (Socransky and Haffajee, 2005).
[b] Organisms of the ''Orange Complex''; the consortium most associated with mild/early stages of disease, whose colonization is thought to be essential for the subsequent succession of the Red complex organisms (Socransky and Haffajee, 2005).

viruses. Herpesviruses were recently suggested to play a significant role in periodontal disease, possibly by disrupting host defenses and facilitating bacterial infection (Slots, 2007). Human immunodeficiency virus (HIV) has been found in saliva, but transmission of HIV from oral secretions is much less common than rectal or vaginal routes (Quiñones-Mateu *et al*., 2003; Sun *et al*., 2005).

Thus, microbial populations of the mouth are numerous, diverse, and site-specific. HDPs are similarly site specific and they comprise significant components of the innate defense secretions that protect all oral tissues and that help regulate resident and pathogenic microbial communities.

III. HDP EXPRESSION IN THE MOUTH

A. Innate defenses in the mouth

Tissues of the oral cavity are constantly exposed to innate defenses derived from saliva, gingival crevicular fluid (GCF), epithelial cells, and neutrophils, and HDPs are significant in all of these, working in synergy with other defense components. Saliva is a multifunctional secretion containing components that contribute to oral buffering, lubrication, enamel remineralization, taste, digestion, and aggregation (e.g., agglutinin and mucins, MUC5B, and MUC7) (Nieuw Amerongen and Veerman, 2002). Constituents that are either directly antimicrobial or interfere with microbial colonization or nutrition include HDPs, secretory IgA, lactoferrin, lysozyme, lactoperoxidase, myeloperoxidase, chitinase, calprotectin, and chromagranin A. Cystatins and Von Ebner glands protein (VEGL) are cysteine proteinase inhibitors and cystatins are also antimicrobial and immunomodulatory. Secretory leukocyte proteinase inhibitor (SLPI) inhibits serine proteinases and additionally displays some antimicrobial and wound-healing properties, and TIMP (tissue inhibitors of metalloproteinases) contribute to regulating extracellular matrix remodeling and turnover through their ability to inhibit matrix metalloproteinases (Nieuw Amerongen and Veerman, 2002).

GCF, a serum-like exudate that flows into the gingival crevice, contains leucocytes, epithelial and bacterial cells, serum proteins, inflammatory mediators, host cell degradation products, and microbial metabolites (Pisano *et al*., 2005). Polymorphonuclear neutrophils are key in periodontal homeostasis and defense (Schenkein, 2006); they account for 90% of the leucocytes in GCF and their concentration increases 15-fold in periodontally diseased sites (Pisano *et al*., 2005). Infiltrating lymphocytes, neutrophils, macrophages, and T cells further secrete cytokines, chemokines, and matrix metalloproteinases into GCF and tissues (Schenkein, 2006). It has been proposed that commensal subgingival plaque bacteria

help regulate the establishment of a protective layer of neutrophils strategically positioned between subgingival plaque bacteria and the junctional epithelium by regulating low levels of expression of intracellular adhesion molecule 1 (ICAM-1), E-selectin, and IL-8 (Dixon *et al*., 2004a).

B. Histatins

Salivary histatins, linear HDPs of 3–5 KDa with high histidine content and strong antifungal activities (Oppenheim *et al*., 1988), account for 2.6% of total salivary proteins (Shimada, 2006) but have not been detected in GCF (Pisano *et al*., 2005). Two human genes encode histatin secretion in parotid, submandibular, and sublingual salivary glands and in the minor von Ebner's glands (Piludu *et al*., 2006). The two primary products, histatin 1 and histatin 3, undergo sequential cleavage, resulting in two salivary fragments of histatin 1 and at least 24 fragments of histatin 3 (Castagnola *et al*., 2004; Helmerhorst *et al*., 2006a). The predominant forms in saliva are histatin 1, 3, and 5, and histatins 1 and 5 have also been detected in the acquired pellicle formed on human enamel *in vitro* and *in vivo* (Schupbach *et al*., 2001; Vitorino *et al*., 2005).

C. Defensins

Defensins are cysteine-containing peptides with three disulfide bonds (Lehrer, 2004). The α-defensins include human neutrophil defensins (HNPs 1–4), which are synthesized in primary neutrophil granules, and HD5 and HD6 produced by Paneth cells and epithelial cells of the genitourinary tract. In the mouth, HNPs1–4 have been detected in gingival junctional epithelium (Dale and Fredericks, 2005). They are not detected *in vitro* in oral keratinocytes culture. Nonetheless, HNP mRNAs are frequently detected in oral tissue and in GCF they reach approximately eight times their salivary concentration, suggesting that neutrophils in GCF are a major source of oral α-defensins (Dale *et al*., 2001; Dunsche *et al*., 2001; Lundy *et al*., 2005; Pisano *et al*., 2005). However, Tao *et al*. (2005) detected HNPs 1–3 in ductal cells of salivary glands and α-defensins have been shown to accumulate within cells that do not themselves synthesize them, for example, T cells and certain epithelial cells (Hazrati *et al*., 2006; Lehrer, 2004).

Expression of the human β-defensin (hBD) peptides, a large group of HDPs that differ from α-defensins in their pattern of disulfide bond formation (Lehrer, 2004), may be constitutive or inducible, and induction depends on the stimulus, host cell type, and differentiation state. hBDs are synthesized by epithelial cells and certain immune cells, and are involved in innate defenses of a wide range of tissues, including the mouth (Devine, 2003). Salivary glands express hBDs 1–3 (Bonass *et al*., 1999; Mathews *et al*., 1999) (Table 8.2–8.4) and neutrophils, macrophages,

TABLE 8.2 Human β-defensin 1 expression in the oral cavity

Cell/tissue type	Response to Stimulus	References
In situ		
Saliva*, submandibular gland, parotid gland, small labial gland Gingiva, tongue, buccal and labial mucosa, floor of mouth Dental pulp* Dental follicle	NA	Bonass *et al*., 1999; Dommisch *et al*., 2005a,b; Dunsche *et al*., 2001, 2002; Mathews *et al*., 1999
Palatine tonsil*, ↓ in recurrent acute tonsillitis	NA	Ball *et al*., 2007
Differentiated layer of stratified epithelium	NA	Dale *et al*., 2001
Granular and spinous layer of gingival epithelia* healthy and chronic periodontitis Inflamed connective tissue	NA	Lu *et al*., 2004, 2005
In vitro		
Oral keratinocytes	↑: IFNγ	Joly *et al*., 2005

Oral keratinocytes	Constitutive: LPS, IL1β, TNFα, PMA, *F. nucleatum* cell wall extract, IL8, IL6, periodontal state, *A. actinomycetemcomitans*, *E. coli*, *F. nucleatum*	Bissell *et al*., 2004; Dale *et al*., 2001; Mathews *et al*., 1999; Ouhara *et al*., 2006; Vankeerberghen *et al*., 2005
Odontoblast	↓: LPS, Pam3CSK4	Veerayutthwilai *et al*., 2007
Oral keratinocytes	↑: PMA, TNFα, *P. gingivalis*, *A. actinomycetemcomitans*	Vankeerberghen *et al*., 2005

Oral keratinocytes were generally obtained from gingival or palatal biopsy or normal oral tissue overlaying the molars.
* mRNA and peptide expression detected.
** peptide expression detected.
↓ : reduced expression.
↑ : increased expression.
PMA: phorbol myristate acetate; LPS: lipopolysaccharide TLR4 ligand; TNFα: tumor necrosis factor α ; IFNγ: Interferon γ; Pam3CSK4: Tripalmitoylated lipopeptide (synthetic bacterial lipoprotein—TLR2-TLR1 ligand); NA: not applicable.

TABLE 8.3 Human β-Defensin 2 Expression in the Oral Cavity

Cell/tissue type	Response to stimulus	References
In situ		
Gingival epithelium (inflamed or non inflamed)	NA	Hosokawa *et al*., 2006
Granular and spinous layer of gingival epithelia* (healthy and chronic periodontitis)	NA	Lu *et al*., 2004, 2005
Inflamed connective tissue		
Saliva**, submandibular gland, small labial gland	NA	Bonass *et al*., 1999; Dommisch *et al*., 2005a,b; Dunsche *et al*., 2001, 2002; Mathews *et al*., 1999
Gingival epithelium (healthy/inflamed)		
Tongue, buccal, labial mucosa, floor of mouth		
Dental pulp*, dental follicle		
Differentiated layer of stratified epithelium	NA	Dale *et al*., 2001
In vitro		
Oral keratinocytes	↑: healthy compared with inflamed	Bissell *et al*., 2004
	↑: HIV, IL-8, IL-6, IL-1β, TNFα, IFNγ, LPS, PMA	Joly *et al*., 2005; Mathews *et al*., 1999; Ouhara *et al*., 2006; Quiñones-Mateu *et al*., 2003; Vankeerberghen *et al*., 2005

	↑: *F. nucleatum* cell wall extract	Dale *et al*., 2001;
	↑: PMA	Krisanaprakornkit *et al*., 2002, 2003
	↑: *S. gordonii*; *P. gingivalis*, *A. actinomycetemcomitans*	Chung and Dale, 2004; Taguchi and Imai, 2006
	↑: *A. actinomycetemcomitans* omp100	Ouhara *et al*., 2006
	↑: *P. gingivalis* Arg-gingipain B	Dommisch *et al*., 2007a
	↑: *A. actinomycetemcomitans*, *F. nucleatum**, *E. corrodens**, *P. gingivalis*, *P. intermedia**, *E. coli*	Hosokawa *et al*., 2006; Vankeerberghen *et al*., 2005
	↑: *C. albicans* hyphae	Feng *et al*., 2005
Odontoblasts	↓: Pam3CSK4 treatment	Veerayutthwilai *et al*., 2007
	↑: LPS treatment	

Oral keratinocytes were generally obtained from gingival or palatal biopsy or normal oral tissue overlaying the molars.
* mRNA and peptide expression detected.
** peptide expression detected.
↓ : reduced expression.
↑ : increased expression.
PMA: phorbol myristate acetate; LPS: lipopolysaccharide TLR4 ligand; TNFα: tumor necrosis factor α ; IFNγ: Interferon γ; Pam3CSK4: Tripalmitoylated lipopeptide (synthetic bacterial lipoprotein—TLR2-TLR1 ligand); NA: not applicable.

TABLE 8.4 Human β-defensin 3 expression in the oral cavity

Cell/tissue type	Response to stimulus	References
In situ		
Gingiva	NA	Dunsche *et al*., 2002; Hosokawa *et al*., 2006
Tongue, buccal mucosa, labial mucosa, floor of mouth, dental follicle		
Palatine tonsil* (↓ in recurrent acute tonsillitis)	NA	Ball *et al*., 2007
Granular and spinous layer of gingival epithelia* (healthy and chronic periodontitis)	NA	Lu *et al*., 2005
In vitro		
Oral keratinocytes	↑: IL-8, IL-6, IL-1β, TNFα, IFNγ, PMA	Joly *et al*., 2005; Ouhara *et al*., 2006; Vankeerberghen *et al*., 2005
	↑: *P. gingivalis*, *A. actinomycetemcomitans*, *F. nucleatum*	Vankeerberghen *et al*., 2005
	↑: HIV	Quiñones-Mateu *et al*., 2003
	↑: *C. albicans* hyphae	Feng *et al*., 2005
Odontoblast	↓: LPS and Pam3CSK4	Veerayutthwilai *et al*., 2007

Oral keratinocytes were generally obtained from gingival or palatal biopsy or normal oral tissue overlaying the molars.
* mRNA and peptide expression detected.
** peptide expression detected.
↓ : reduced expression.
↑ : increased expression.
PMA: phorbol myristate acetate; LPS: lipopolysaccharide TLR4 ligand; TNFα: tumor necrosis factor α ; IFNγ: Interferon γ; Pam3CSK4: Tripalmitoylated lipopeptide (synthetic bacterial lipoprotein—TLR2-TLR1 ligand); NA: not applicable.

monocytes, and dendritic cells synthesize hBDs 1 and 2 (Duits *et al*., 2002). Gingival epithelial cells secrete hBDs 1, 2, and 3 and gene expression studies indicate up to 10 putative hBD genes may be expressed in gingival epithelium (Premratanachai *et al*., 2004). hBDs are also expressed by tongue and buccal epithelial cells and they contribute to the defense of deeper oral tissues as they are expressed within dental pulp and by odontoblasts (Dommisch *et al*., 2005b; Dunsche *et al*., 2002; Veerayutthwilai *et al*., 2007) (Tables 8.2–8.4).

In oral tissue, as in other tissues, hBD 1 expression is constitutive regardless of inflammation and disease state and some *in vitro* studies of oral keratinocytes found constitutive expression in spite of diverse pro-inflammatory stimuli (Table 8.2). However, studies of hBD 1 expression *in vitro* present some contradictions, and expression probably depends on cell type, stimulus concentration, and duration. Inducible expression has been reported in oral keratinocytes after IFNγ, PMA, TNFα, *P. gingivalis*, and *A. actinomycetemcomitans* stimulation (Joly *et al*., 2005; Vankeerberghen *et al*., 2005) and hBD 1 expression was decreased in odontoblasts after stimulation with TLR agonists (Veerayutthwilai *et al*., 2007).

Gene expression and protein secretion of hBD 2 and hBD 3 are modulated by various bacterial/viral/fungal components, injury, and inflammatory stimuli, and hBD 2 is also upregulated in monocytes and macrophages exposed to bacteria, LPS, or IFNγ (Duits *et al*., 2002; Fang *et al*., 2003).

In situ analyses of hBD 2 and hBD 3 expression have reported low basal expression of these peptides in diverse tissues of the oral cavity (Tables 8.3 and 8.4). hBD 2 expression is associated with differentiation, as its mRNA was detected in the differentiated layer of the stratified epithelium or *in vitro*, in involucrin positive epithelial cells (Dale *et al*., 2001). hBD 3 is expressed in the basal and spinous layers of gingival epithelial cells, not only in keratinocytes but also in Langerhans and Merkel cells (Lu *et al*., 2005). Defensins are not expressed in connective tissue (Dale *et al*., 2001) except in inflamed connective tissue of periodontal subjects due to keratinocyte activities in the pocket site (Lu *et al*., 2004).

In vitro studies of oral keratinocytes have shown that hBD 2 and 3 expression is induced in response to cytokines, TLR agonists, bacteria/bacterial components, PMA, viruses, and fungi (Tables 8.3 and 8.4). Transcriptional stimulation is regulated with the expression of other components of innate immunity, such as pro-inflammatory cytokines (IL-1β, IL-8, IL-6, and TNFα) released during TLR activation (Dommisch *et al*., 2007b; Ouhara *et al*., 2006; Veerayutthwilai *et al*., 2007). Therefore, it is also possible that these cytokines may indirectly account for the regulation of β-defensin expression in an autocrine manner.

Key signaling pathways that regulate downstream innate immune gene functions are activated or modulated by hBDs or involved in the regulation of hBD expression. Several studies have demonstrated

the NF-κB and AP-1 dependent activation of hBD 2 expression in intestinal cells and in keratinocytes (Wehkamp *et al*., 2007) and the transcription factor, STAT3, is also involved in hBDs 2–3 expression in keratinocytes in response to IL-22 (Wolk *et al*., 2004). In oral keratinocytes, P38, JUNK, ERK, and NF-κB are the main pathways used to activate hBD 2 expression in response to bacteria/bacterial components (Table 8.3), and different organisms utilize distinct pathways (Chung and Dale, 2004). Alternate pathways such as JACK-STAT are used in oral keratinocytes to induce hBD 1 expression in response to IFNγ (Joly *et al*., 2005) and the type 2 protease activated receptor (PAR2) signaling pathway was activated in response to *P. gingivalis* proteases to induce hBD 2 (Dommisch *et al*., 2007a). Therefore, transcriptional regulation of hBDs is regulated and coordinated with the expression of other entities of innate immunity.

D. Cathelicidin LL-37

Cathelicidins share a highly conserved pro-region and are synthesized in a wide range of mammalian species although only one endogenous cathelicidin, LL-37/hCAP18, has been identified in humans (Lehrer and Ganz, 2002). It is found at high concentrations in its unprocessed form (hCAP18) in granules of neutrophils and is processed extracellularly upon degranulation and release by proteinase-3 (Sorensen *et al*., 2001). LL-37 is also produced by a variety of epithelial cells and secreted into body fluids (Bals and Wilson, 2003; Devine, 2004; Lehrer and Ganz, 2002). Its expression increases in response to 1,25-dihydroxyvitamin D(3) and inflammatory conditions but the molecular mechanisms regulating its expression remain largely unknown.

In the oral cavity, LL-37 expression (Table 8.5) has been detected in connective tissue, junctional epithelium, palatine tonsil, tongue, buccal mucosa, inflamed gingival tissue, saliva, salivary gland, and submandibular glands (Ball *et al*., 2007; Frohm Nilsson *et al*., 1999; Hosokawa *et al*., 2006; Murakami *et al*., 2002a; Tao *et al*., 2005; Woo *et al*., 2003). *In vitro* studies of LL-37 expression are equivocal, as Dale *et al*. (2001) reported constitutive expression in response to diverse pro-inflammatory stimuli, whereas others have reported inducible expression of LL-37 in response to bacteria, IL8, and IL-1β (Table 8.5). The 5′ flanking sequence of the cathelicidin coding sequence (CAMP) has several potential consensus sequences for transcription factors, including NF-κB, NF-IL6, acute phase response factor, and IFN-γ response element (Gudmundsson *et al*., 1996; Tomasinsig and Zanetti, 2005). Thus, although the molecular mechanisms regulating LL-37 expression remain largely unknown, LL-37 transcriptional regulation and activities appear to be coordinated by key regulators of innate immunity and with the expression of innate immune

TABLE 8.5 LL37 expression in the human oral cavity

Cell/tissue type	Response to stimulus	References
In situ		
Connective tissue*, junctional epithelium* (possibly in infiltrated neutrophils)	NA	Dale *et al*., 2001
Palatine tonsil* (↓ in recurrent acute tonsillitis)	NA	Ball *et al*., 2007
Saliva**	NA	Frohm Nilsson *et al*., 1999; Murakami *et al*., 2002
Tongue*, buccal mucosa* (basal layer of epithelium)		
Inflamed gingival tissue* (↑ proportional to severity of disease)	NA	Hosokawa *et al*., 2006
Salivary glands* (↑ chronic sialadenitis), submandibular glands*	NA	Tao *et al*., 2005; Woo *et al*., 2003
In vitro		
Oral keratinocytes	Constitutive: bacterial extract, PMA, TNFα	Dale *et al*., 2001
	IL-8, IL-1β, *A. actinomycetemcomitans*	Ouhara *et al*., 2006
	*A. actinomycetemcomitans**, *F. nucleatum**, *E. corrodens**, *P. gingivalis*, *P. intermedia**	Hosokawa *et al*., 2006

Oral keratinocytes were generally obtained from gingival or palatal biopsy or normal oral tissue overlaying the molars.
* mRNA and peptide expression detected.
** peptide expression detected.
↓ : reduced expression.
↑ : increased expression.
PMA: phorbol myristate acetate; LPS: lipopolysaccharide TLR4 ligand; TNFα: tumor necrosis factor α ; IFNγ: Interferon γ; Pam3CSK4: Tripalmitoylated lipopeptide (synthetic bacterial lipoprotein—TLR2-TLR1 ligand); NA: not applicable.

genes. This is consistent with its multiple and complex mechanisms of action regarding innate immune functions (Mookherjee *et al*., 2006).

IV. FUNCTIONS OF HDPs IN THE MOUTH

A. Antibacterial functions

Most HDPs exert their effects through interactions with eukaryotic and prokaryotic membranes, and they also bind avidly to bacterial LPS and lipoteichoic acids (LTA). Although the lethal event for many HDPs is destabilization and permeabilization of the cytoplasmic membrane, some peptides reach internal cytoplasmic targets; thus, the precise mechanism of killing is dependent on peptide and membrane structure, as well as the responses of the target organism (Brogden, 2005; Devine and Hancock, 2002).

Defensins adopt triple-stranded β-pleated sheet structures in aqueous environments and their amphiphilicity facilitates spontaneous insertion into membranes, wherein they form voltage-dependent channels. Although defensins can depolarize cytoplasmic membranes, this may not be their lethal bactericidal action (Brogden, 2005; Lehrer, 2004). LL-37 adopts an amphipathic α-helical structure upon interaction with membranes and a variety of mechanisms have been suggested for subsequent membrane disruption (Brogden, 2005). The primary antimicrobial actions of histatins are antifungal, although they exhibit some antibacterial activity (Mackay *et al*., 1984) and their activity against Gram-positive bacteria appears to be dependent on zinc binding (Rydengard *et al*., 2006).

1. Activities against oral bacteria

The relative activities of defensins against oral bacteria follows similar trends to those observed with other organisms: HNP1 and HNP2 have greater activity than HNP3, hBD 2 is more bactericidal than hBD 1, and hBD 3 has greater potency and a broader spectrum of activity than either hBD 1 or 2 (Brissette *et al*., 2004; Joly *et al*., 2004; Maisetta *et al*., 2003; Ouhara *et al*., 2005). LL-37 has activity against a range of Gram-positive and Gram-negative oral bacteria (Ouhara *et al*., 2005; Tanaka *et al*., 2000). Histatins inhibit the growth of oral streptococci (Payne *et al*., 1991). Histatin 5 inhibits *P. gingivalis* gingipains (Gusman *et al*., 2001), which mediate a variety of adhesive interactions as well as proteolysis (Curtis *et al*., 2001) and this may explain the inhibitory effects of histatin 5 on *P. gingivalis* growth, hemagglutination, and coaggregation (Murakami *et al*., 1991; Murakami *et al*., 1992). *A. actinomycetemcomitans* produces a potent leukotoxin, which is also inhibited by histatin 5 (Murakami *et al*., 2002b).

There is not a consistent relationship between killing of oral bacteria by HDPs and relative pathogenicity. Although Shelburne *et al*. (2005) found *F. nucleatum* ATCC 25586 to be resistant to 100 μg/ml hBDs 1, 2, 3, and 4, others found multiple *F. nucleatum* strains were killed by hBD 3 and by LL-37, as were caries-associated and commensal streptococci and strains of *A. actinomycetemcomitans* (Ouhara *et al*., 2005; Tanaka *et al*., 2000). There was considerable interstrain variation in sensitivity of some species, highlighting the importance of studying multiple strains. *P. gingivalis* and *Prevotella intermedia* strains were sensitive to 20 μg/ml of a 27 amino acid variant of LL-37 (Isogai *et al*., 2003) and were relatively insensitive to hBD 3 (Ouhara *et al*., 2005). However, *P. gingivalis* ATCC 33277 was highly sensitive to hBDs 1–4 (Shelburne *et al*., 2005). In contrast, oral spirochetes display a high level of resistance to hBDs (Brissette *et al*., 2004).

The *in vivo* antibacterial activities of HDPs are uncertain, although HDPs are present in saliva in concentrations sufficient to be antibacterial (Tao *et al*., 2005) and normal expression of hBD 2 in oral epithelial tissue has been suggested to be about 0.04 μg/mg (Sawaki *et al*., 2002). HDPs are significantly inhibited by physiological salt concentrations, with the exception of hBD 3 (Hancock and Sahl, 2006; Joly *et al*., 2004; Ouhara *et al*., 2005). However, hBD 3 activity against *A. actinomycetemcomitans*, but not *S. mutans,* was inhibited by serum and saliva (Maisetta *et al*., 2005) as was the activity of LL-37 (Ouhara *et al*., 2005; Tanaka *et al*., 2000). Studies of LL-37 in which activity has been assayed in broth (Altman *et al*., 2006; Guthmiller *et al*., 2001) have yielded high levels of resistance compared with assays in buffer or dilute medium. This confirms many studies of nonoral bacteria, which indicate numerous HDPs are inhibited by components of bacteriological media, and again highlights the importance of assay design in developing screening or experimental procedures for HDPs.

The effects of environment and medium composition may not only affect HDP function but also bacterial phenotype. The activities of LL-37 and hBD 2 were increased by growth of bacteria in the presence of bicarbonate, which modulated bacterial gene expression and induced less susceptible phenotypes (Dorschner *et al*., 2006). Bicarbonate is significant in the mouth, for example, in the buffering capacity of saliva (Nieuw Amerongen and Veerman, 2002) but its role in regulating gene expression in oral bacteria has not been studied. Short-term reductions in sensitivity to hBDs 1–4, as well as to heat or peroxide stress, was induced in a sensitive strain of *P. gingivalis* by prior exposure of the organism to hBDs, a phenomenon that would be highly significant if it occurs *in vivo* (Shelburne *et al*., 2005). Bacterial phenotype is also affected by growth within biofilms, and few studies of the activities of HDPs against oral bacteria have considered that these organisms exist *in vivo* in multispecies

biofilms where they benefit from community-based living and also may express more resistant phenotypes.

2. Resistance mechanisms

Resistance to HDP killing is determined by properties such as the charge density and structure of LPS, lipid composition, and electrochemical potential of the cytoplasmic membrane, responses of bacterial cells to environmental conditions, and peptide transport and efflux mechanisms (Devine and Hancock, 2002; Peschel and Sahl, 2006). LPS structure is of primary importance in determining susceptibility. *P. gingivalis* LPS displays environmentally regulated variable lipid A acylation, resulting in a mixture of chemotypes that interact with TLR4 or TLR2 (Dixon and Darveau, 2005), and the repeating unit of *P. gingivalis* O-polysaccharide is highly phosphorylated (Paramonov *et al*., 2001); these properties may be significant in moderating HDP binding. Cell surface decoration with host-derived phosphorylcholine is displayed by a range of oral organisms, for example, streptococci, *Neisseria* spp., *Actinomyces* spp., *Fusobacterium* spp., and such host-mimicry may reduce sensitivity to LL-37 (Devine, 2003). In staphylococci the *dlt* operon, which many oral Gram-positive species also possess, mediates D-alanyl esterification of teichoic acids, which decreases cell wall negative charge and reduces HDP binding (Peschel and Sahl, 2006). It may be significant to *in vivo* pathogenicity that many of the cell surface alterations that affect bacterial resistance to HDPs also have significant impact on interactions with host cell receptors and the signaling pathways that are initiated (Devine, 2003, 2004).

Bacterial production of extracellular and membrane-bound proteases may degrade HDPs or peptide uptake and transport systems can bind and divert HDPs from target sites. Many oral anaerobes are strongly proteolytic and strains of *P. gingivalis* and *Prevotella* spp. secrete proteases that cleaved and inactivated HDPs while representative oral streptococci, actinomyces, and *Rothia dentocariosa* did not (Devine *et al*., 1999). Resistance of *T. denticola* to hBDs was not related to its ability to produce proteases, but is more likely related to the fact that it does not possess a typical negatively charged LPS (resulting in poor binding of HDPs) and to its possession of peptide efflux systems (Brissette and Lukehart, 2007).

B. Antifungal activities

Many HDPs exert antifungal activity and their mechanisms of action against *C. albicans* may be via: (i) membrane permeabilization followed by cell necrosis; (ii) uptake and inhibition of respiration or of oxygen free-radical scavengers followed by increased production of reactive oxygen species (ROS) and cell necrosis or apoptosis; (iii) uptake and promotion of

ATP release and cell death by poorly defined mechanisms; (iv) activation of signal transduction pathways that result in increased intracellular cAMP and apoptosis (Helmerhorst and Oppenheim, 2004).

Histatins are efficient antifungal peptides and the best studied HDPs in this respect. Histatin 5 binds to specific *C. albicans* membrane receptors (heat shock proteins Ssa1 and Ssa2) and, once bound, it utilizes the yeast Trk1p potassium transporter and interferes with mitochondrial respiratory processes, disrupts the cell cycle, and promotes generation of ROS, and death ensues via cell necrosis (Baev *et al*., 2004; Helmerhorst and Oppenheim, 2004; Vylkova *et al*., 2006). Defensins possess anti-candidal activities and β-defensins also inhibit binding of *C. albicans* to oral epithelial cells (Feng *et al*., 2005). HNP1, hBD 2, and hBD 3 bind to unidentified receptors (not Ssa1 or Ssa2 proteins) and do not require Trk1p, and hBD 3 uses pathways that are distinct to those employed by other defensins or histatin 5 to induce cell death (Vylkova *et al*., 2006, 2007). hBD 3-mediated killing of *C. albicans*, in contrast to its antibacterial activity, was highly salt-sensitive and inhibition of hBD 2-mediated killing was specific to Mg^{2+} and Ca^{2+} ions (Vylkova *et al*., 2007). Differences in observations regarding the anti-candidal activities of hBDs may reflect the source of the peptide: although the reason is not clear, commercially supplied hBD 3 was 6–14 times more fungicidal than hBD 2 (Vylkova *et al*., 2006) while recombinant hBD 2 was 10 times more active against *C. albicans* than recombinant hBD 3 (Feng *et al*., 2005). LL-37 kills *C. albicans* via an, as yet, poorly defined mechanism, which involves significant membrane disruption and leakage of cell components including nucleotides and proteins (den Hertog *et al*., 2005). Resistance of yeasts to the actions of HDPs has been observed, particularly in *Candida glabrata*. This species is emerging as an important oral pathogen, and many strains are resistant to antifungal drugs as well as to histatins and defensins through, as yet, poorly defined mechanisms (Helmerhorst *et al*., 2006b; Joly *et al*., 2004; Li *et al*., 2007; Vylkova *et al*., 2007).

C. Antiviral activities

Infectivity of herpesviruses and other enveloped viruses is inhibited by HNPs 1–3 (Daher *et al*., 1986), and hBD 3, but not hBD 1 or hBD 2, inhibits HSV infection (Hazrati *et al*., 2006). HNPs 1–3 also inhibit papilloma virus post-entry into target cells by blocking viral escape from endocytic vesicles (Buck *et al*., 2006). Defensins display lectin-like binding activities and bind to some viral surface glycoproteins and to CD4 receptors (Hazrati *et al*., 2006; Lehrer, 2004). Thus, defensins can defend against virus infection by directly acting on virus particles or through acting on target cells pre or postviral entry (Klotman and Chang, 2006; Lehrer, 2004).

D. Non-antimicrobial functions

1. Histatins

Histatins are multifunctional within the mouth and also affect processes in the gut, as they form stable complexes with dietary polyphenols, contributing alongside proline rich proteins to salivary detoxification of these damaging plant secondary metabolites (Cai and Bennick, 2006; Shimada, 2006). Histatins are present in pellicle (Nieuw Amerongen and Veerman, 2002). Histatin 1 has a high affinity for hydroxyapatite and inhibits *S. mutans* adhesion to hydroxyapatite *in vitro* (Shimotoyodome *et al*., 2006). As it is also thought to contribute to enamel remineralization and protection from demineralization (Vitorino *et al*., 2005), histatin 1 has the potential to protect against processes involved in dental caries formation. Cytokine induction in gingival fibroblasts by outer membrane proteins of *P. gingivalis* was inhibited by histatin 5 (Imatani *et al*., 2000), and this peptide also inhibits host matrix metalloproteinases (Gusman *et al*., 2001). Thus, histatin 5 has the potential to modulate inflammatory processes and tissue destruction that contribute to periodontal disease, although histatins have not been detected in GCF so their ability to influence events in the subgingival domain is limited.

2. Defensins

Defensins display numerous immunomodulatory properties (Niyonsaba *et al*., 2006; Yang and Oppenheim, 2004; Yang *et al*., 2002). They can act as chemokines, chemoattracting monocytes, neutrophils (memory and naïve) T cells, and immature dendritic cells (DCs). They also induce mast cell degranulation and release histamine and prostaglandin D(2). Moreover, neutrophil defensins may modulate inflammatory responses through regulation of cytokine production and adhesion molecule expression. Similarly, β-defensins are involved in cytokine/chemokine production and participate in wound healing and tissue remodeling (Niyonsaba *et al*., 2007; Varoga *et al*., 2005). Defensins also act at the interface of innate and adaptive immunity. Mouse β-defensin 2 has been shown to act directly on immature DCs as an endogenous ligand for TLR4, inducing upregulation of costimulatory molecules and DC maturation and triggering Th1 polarized adaptive immune responses *in vivo* (Yang *et al*., 2002).

There is also evidence that human defensins exert immune regulatory functions in the context of the oral cavity. In odontoblast-like cells, recombinant hBD 2 downregulated expression of hBD 1 but it induced expression of IL-6, IL-8, and $cPLA_2$ in a dose- and time-dependent manner (Dommisch *et al*., 2007b). hBD 2 also stimulated odontoblast differentiation (Shiba *et al*., 2003), and promoted migration of human oral epithelial cells (our unpublished data). A regulatory role for defensins in generating

mucosal adaptive immune responses to various foreign antigens in the oral cavity has also been demonstrated, as HNPs and hBDs, coadministered intranasally with the antigen ovalbumin, induced unique immune responses in the oral cavity of a murine model (Brogden *et al*., 2003).

3. LL-37

The physiological importance of LL-37 in defense of the oral cavity is determined not only by its antimicrobial properties, but also by its broad range of immunomodulatory functions. LL-37 stimulates the expression, in monocytes/macrophages, of a wide variety of genes involved in the innate immune response, including those encoding chemokines (e.g., IL-8 and MCP-1), differentiation factors, and anti-inflammatory cytokines (Scott *et al*., 2002). LL-37 is also directly chemotactic for human neutrophils, monocytes, T cells, and mast cells (Niyonsaba *et al*., 2006; Yang and Oppenheim, 2004). It has further been shown to modulate the TLR-induced inflammatory responses in monocytes (Mookherjee *et al*., 2006) and to protect mice and rats against endotoxemia/sepsis induced by pure LPS (Fukumoto *et al*., 2005; Scott *et al*., 2002). LL-37 has a variety of other functions in immunity, including the promotion of mast cell histamine release, stimulation of wound healing and angiogenesis (Bals and Wilson, 2003; Niyonsaba *et al*., 2006), modulation of dendritic cell differentiation (Davidson *et al*., 2004), and the regulation of apoptosis of epithelial cells or neutrophils (Barlow *et al*., 2006). Thus, LL-37 appears to be an important component of both the phagocyte and epithelial defense systems in humans.

Few studies have been devoted specifically to the immunomodulatory functions of LL-37 in the oral cavity. However, LL-37 was observed to regulate submandibular gland responses (Pochet *et al*., 2006) and a truncated C-terminal LL-37 ($hCAP18_{109–135}$) induced apoptosis of oral squamous cell carcinoma SAS-H1, but not normal cells, in a caspase independent manner (Okumura *et al*., 2004). Like hBDs, LL37 promotes migration of human oral epithelial cells, suggesting it has potential wound healing properties in the oral cavity (our unpublished data).

V. ROLES OF HDPs IN ORAL HEALTH AND DISEASE

The often quoted estimate that bacterial cells colonizing the body outnumber human cells by a ratio of 10:1 (Savage, 1977) highlights an apparent paradox; that these resident populations are tolerated while the host maintains the ability to respond effectively to pathogenic microorganisms. The hypothesis that HDPs are important in maintaining the homeostatic balance between a host and its highly diverse resident populations

(Boman, 1995) has gained momentum with increased knowledge of their expression, evolution, and antimicrobial and immunomodulatory properties (Devine, 2004; Peschel and Sahl, 2006; Weinberg *et al*., 1998). It has become clear that resident microbial populations are in a dynamic relationship with the host, and their presence and control is essential to health and development, so the proposed regulatory role of HDPs is vital. There is also little doubt that HDPs are significant in the host response and defense against infection but their roles are difficult to dissect when they are expressed at sites that are usually heavily colonized (e.g., oral cavity), compared with normally sterile or sparsely colonized tissue (e.g., lung, bladder).

A. Microbial induction of oral HDP expression

Both pathogenic and commensal oral microorganisms would benefit from either a failure to induce HDP synthesis or by an ability to downregulate their expression. Studies of this kind, which have mainly concentrated on organisms associated with periodontal diseases, are complicated by the polymicrobial nature of the diseases, which additionally arise from the normal microbiota, making host–microbe relationships difficult to define. For example, *F. nucleatum* has been used in some studies as a representative oral commensal (Chung and Dale, 2004; Krisanaprakornkit *et al*., 2000) because it is found in healthy and diseased subgingival sites with similar prevalence, and it is an important organism in plaque formation. On the other hand, it has been used as a representative periodontal pathogen (Ji *et al*., 2007) because it is a member of the ''Orange complex'' that is proposed as an essential precursor to severe disease and emergence of the ''Red complex'' organisms (Socransky and Haffajee, 2005), and because it displays properties associated with pathogenicity. *F. nucleatum* can also facilitate transport into buccal epithelial cells of coaggregation partners that are otherwise unable to invade (Edwards *et al*., 2006). Recent developments in better characterization of the organisms that are associated only with healthy subgingival plaque and those associated with early disease should facilitate progress in this area and allow rational selection of a greater range of species clearly associated with health or with distinct stages of disease.

To date, studies have shown that both oral commensals and organisms associated with periodontal disease are able to induce HDPs (Tables 8.2–8.5). However, there has been considerable variation in findings, which is well illustrated by studies of *P. gingivalis*. Cell wall extracts of *P. gingivalis* ATCC 33277 did not induce expression of hBD 2 in primary oral keratinocytes from healthy gingiva, while those of *F. nucleatum* did (Krisanaprakornkit *et al*., 2000). Subsequent studies showed that whole cells of ATCC 33277 (and two other *P. gingivalis* strains) did

induce expression of hBD 2 in similarly isolated primary cells, and this expression was due largely to the activation of PAR2 by the *P. gingivalis* arginine-specific gingipain (Chung and Dale, 2004; Dommisch *et al*., 2007a). In studies using epithelial cells derived from periodontal pockets of people with severe periodontitis undergoing surgery, *P. gingivalis* ATCC 33277 induced hBD 2 expression (Taguchi and Imai, 2006) but five other strains of *P. gingivalis* of varied capsular types induced neither hBD 2 or hBD 4 (Vankeerberghen *et al*., 2005). These five strains did, however, induce expression of hBD 1 and, to varying extents, hBD 3. Using an immortalized oral epithelial cell line, a strain of *P. gingivalis* induced hBD 2 and hBD 3 but this ability was dependent on cell density, with lower multiplicity of infection (MOI) having greater inducing effects (Ji *et al*., 2007); Chung and Dale (2004) found that high MOIs of *P. gingivalis* were cytotoxic. Thus, induction of hBDs by oral epithelial cells appears more often dependent on bacterial strain and host cell type, and on experimental conditions, than on relative pathogenicity of the stimulating bacteria.

Primary gingival epithelial cells derived from healthy tissue were induced to express only low levels of hBD 2 by *A. actinomycetemcomitans* (Chung and Dale, 2004), although expression was induced strongly by killed cells of strain Y4 (Noguchi *et al*., 2003). hBD 3, but not hBD 2, was induced by washed live cells of JP2 (Feucht *et al*., 2003). Vankeerberghen *et al*. (2005) found that induction of hBD 1–4 by five strains of *A. actinomycetemcomitans* was strain, and possibly serotype, dependent. *F. nucleatum* induced hBD 2 and hBD 3 expression in primary gingival cells isolated from healthy and diseased sites (Chung and Dale, 2004; Vankeerberghen *et al*., 2005), but induced hBD 2, hBD 3, and LL-37 in an immortalized cell line only at a high MOI (Ji *et al*., 2007). A strain of the oral commensal *S. gordonii* induced hBD 2 expression in primary gingival epithelial cells (Chung and Dale, 2004) but at a similar MOI did not induce hBDs 1–3 or LL-37 (Ji *et al*., 2007). It has been suggested that oral commensals and opportunistic pathogens utilize different pathways for hBD 2 induction, as *Streptococcus gordonii* and *F. nucleatum* utilized P38 and JUNK and not NF-κB to induce hBD 2 expression, whereas *P. gingivalis* and *A. actinomycetemcomitans* utilized P38, JUNK, and NF-κB (Chung and Dale, 2004; Krisanaprakornkit *et al*., 2002; 2003). Confirmation of this assertion requires the study of more organisms that are truly oral commensals and more strains of putative periodontal pathogens.

C. albicans induced hBDs 1–3 expression, and this was dependent on expression of hyphae and subsequent invasive infections of the filamentous form inhibited hBD expression (Feng *et al*., 2005; Lu *et al*., 2006). Regulation of expression of novel hBDs by *Candida* spp. was strain/species specific and cell donor subject specific, both up and downregulation of specific genes was seen (Premratanachai *et al*., 2004).

B. Expression in oral health and disease

1. Abnormal expression of HDPs

A marked association between oral disease and a specific deficiency in HDPs was observed in studies of patients with morbus Kostmann disease (Putsep *et al*., 2002). This is a severe congenital neutropenia, one manifestation of which is early onset periodontitis, an aggressive localized form of periodontal disease. People with morbus Kostmann have a deficiency in expression of neutrophil LL-37, resulting in only 1–2% of normal plasma levels and low levels in saliva, and reduced expression of HNPs. However, the aggressive periodontitis associated with this deficiency may be a result primarily of the neutrophil defect, rather than the defect in LL-37 synthesis *per se*. Functional polymorphonuclear neutrophils are key in periodontal homeostasis and defects in neutrophil chemotaxis have previously been shown to be linked to susceptibility to aggressive localized periodontitis (Schenkein, 2006). *A. actinomycetemcomitans* is strongly associated with this form of disease and its virulence is dependent on a potent leukotoxin (Fine *et al*., 2006).

Single nucleotide polymorphisms (SNPs) have been detected in the hBD 1 gene and some studies have examined their potential association with disease. In people with type I diabetes, who are prone to *Candida* infections and periodontal disease, a hBD 1 gene SNP (-44, C $\rightarrow$ G) was associated with decreased carriage of *Candida* spp. (Jurevic *et al*., 2003), indicating a potential protective role for this SNP. Subsequent studies found no correlation between the same SNP and protection from, or development of, caries or early onset localized periodontal disease (Boniotto *et al*., 2004; Tao *et al*., 2005). Many more studies are required to understand the roles, if any, of HDP SNPs in oral health and disease.

2. Caries

Saliva contains antimicrobial concentrations of a range of HDPs that might be expected to influence development of caries. It has been stated that there is no proven relationship between caries experience and activity of any salivary antimicrobial protein (Dodds *et al*., 2005). However, a correlation was observed between high levels of histatins 1 and 5 in saliva and pellicle, and low caries experience (Vitorino *et al*., 2005). Histatins in saliva from the caries-free group were more highly bound within salivary complexes than histatins in saliva from the caries-prone group, and the caries-prone group also showed more evidence of proteolytic degradation of salivary proteins and peptides. Tao *et al*. (2005) found a great deal of intersubject variation in concentrations of HDPs in saliva. Nonetheless, high levels of salivary HNPs 1–3 were associated with low caries experience and there was a trend towards higher levels of LL-37 in caries-free children.

3. Periodontal disease

Studies examining expression of HDPs in gingival tissues have produced conflicting results in terms of increased or decreased expression in samples from periodontally diseased sites compared with healthy tissue (Bissell *et al*., 2004; Dommisch *et al*., 2005a; Dunsche *et al*., 2002; Hosokawa *et al*., 2006; Lu *et al*., 2005). mRNA and peptide expression do not necessarily correlate (Hosokawa *et al*., 2006; Lu *et al*., 2005), indicating post-transcriptional or post-translational events are influential. Decreased LL-37 expression has been associated with acute tonsillitis (Ball *et al*., 2007); however, the significance of LL-37 expression in inflammatory conditions such as periodontitis can be difficult to assess because of neutrophil accumulation (Hosokawa *et al*., 2006; Woo *et al*., 2003).

It may be too late to understand the processes that have contributed to the disease process by the time a site is showing overt clinical signs of periodontal disease and periodontal pockets have developed; by then the site is in crisis and profound local tissue damage has occurred alongside significant deregulation of inflammatory pathways. Some studies have compared hBD peptide expression in tissues from inflamed periodontal pockets and from noninflamed tissue adjacent to inflamed sites, as well as noninflamed tissue from subjects with no periodontal disease (Lu *et al*., 2004, 2005). Noninflamed tissue from both sets of subjects expressed similar levels of hBD 1 and these were lower than in inflamed pocket samples. On the other hand, hBD 2 levels were significantly higher in samples from the healthy subjects than in either noninflamed or inflamed tissues from subjects with periodontitis. It is interesting to speculate about the significance of this; it may indicate that in health hBD 2 production is consistently stimulated by the normal microbiota, but individuals with low levels of normal expression of hBD 2 may be predisposed to development of periodontal disease because they cannot sufficiently control the resident microbial populations, or periodontal disease may depress baseline hBD 2 expression in mucosa adjacent to inflamed diseased sites.

Further studies of the distribution of hBDs in gingival tissues revealed hBD 1 and 2 to have similar patterns of expression while hBD 3 was expressed in the basal layers in health and in the basal and spinous layers in periodontal disease (Lu *et al*., 2004, 2005). The authors proposed that hBDs 1–3 function in an orchestrated manner to enhance gingival defense and that the localization of expression of hBD 3 in health in the basal layers of gingival epithelium is strategic to its role in maintaining homeostasis through its ability to promote adaptive immune responses and through its antimicrobial properties.

4. Candida infections

The primary role of histatins *in vivo* has not been elucidated (Nieuw Amerongen and Veerman, 2002) and, although they are potent antifungal peptides, the relationship between histatin expression and candidal

disease is unclear. Although one study found that individuals whose oral mucosa were colonized by *Candida* spp. had reduced salivary flow and lower salivary histatin concentrations compared with those who were uncolonized (Jainkittivong *et al*., 1998), another observed that concentrations of histatins and lysozyme were higher in individuals who suffered from recurrent candidiasis compared with controls (Bercier *et al*., 1999). Decreased hBDs 1–2 peptide secretion was observed in saliva of patients with oral candidiasis when compared with saliva of healthy patients (Tanida *et al*., 2003). Thus, the limited studies carried out examining HDP expression in relation to *Candida* infections are equivocal.

5. HIV

HIV-1 particles induced expression of hBD 2 and hBD 3 in normal human gingival epithelial cells (Quiñones-Mateu *et al*., 2003). In biopsy tissue from healthy individuals and HIV-positive volunteers HNP1–3 expression was similar regardless of HIV status, although hBD 2 was more strongly expressed in epithelial tissues of healthy HIV-negative controls, which probably reflected the HIV immunodeficiency (Sun *et al*., 2005). The fact that HIV is present in the mouth but rarely transmitted by an oral route has led the authors of these studies to propose that oral defenses may be important in natural resistance to this disease. However, the lack of salivary transmission may equally be due to relative lack of ingress of the virus into blood or deeper tissues through oral practices compared with ''high risk'' sexual practices.

VI. THERAPEUTIC APPLICATIONS

When first isolated, HDPs were the only new class of antibiotic to be described for decades. Since then they have attracted attention as potential novel antimicrobial agents with particular emphasis on topical application (Falla and Zhang, 2004; Hancock and Sahl, 2006), which makes oral diseases attractive targets. There have been some setbacks in the path of HDPs to clinical use, including the failure of the first magainin-derived peptide to reach clinical trials (Falla and Zhang, 2004; Hancock and Sahl, 2006). Nonetheless, driven by the shortage of novel antibiotics and increased levels of resistance, a number of companies have continued to pursue the development of HDPs as antimicrobial agents. In addition, their immunomodulatory and wound-healing properties are being exploited to develop nonantimicrobial anti-infective agents.

There has been a large number of studies demonstrating *in vitro* activity of natural or synthetic peptides that are proposed to be promising for development as novel agents for use against oral diseases. For

example, analogues of hBDs, LL-37, and other mammalian cathelicidins as well as peptides from frog skin (magainin and dermaseptin) have been designed with more potency against periodontal pathogens (Altman *et al*., 2006; Genco *et al*., 2003; Guthmiller *et al*., 2001; Isogai *et al*., 2003; Maisetta *et al*., 2005; Porat *et al*., 2006). Histatin analogues have been synthesized with increased antimicrobial activity and resistance to proteases (Giacometti *et al*., 2005; Groenink *et al*., 2003). The natural activities of histatin 5 have also been exploited in the design of short peptides with potential for periodontal therapy that mimic the histatin gingipain cleavage site and inhibit *P. gingivalis* gingipains, hemagglutination, heme acquisition, and coaggregation (Kadowaki *et al*., 2004). Short peptides derived from salivary mucin have been suggested as candidates for development to treat candidiasis and caries because of their *in vitro* activities (Muralidharan and Bobek, 2005; Wei *et al*., 2006) and natural and synthetic cathelicidins displayed activity against a wide range of clinical *Candida* isolates (Benincasa *et al*., 2006). Analogues of cecropin B, dermaseptin, synthetic histatin-lysine polymers, and novel peptides from synthetic combinatorial libraries also have potentially useful anti-caries activities (Altman *et al*., 2006; Concannon *et al*., 2003; Hao *et al*., 2005; He *et al*., 2007; Zhu *et al*., 2006). A novel approach of fusing an antimicrobial peptide domain with a *S. mutans* competence stimulating peptide has generated a peptide with targeted *in vitro* activity against *S. mutans* (Eckert *et al*., 2006). Given the polymicrobial nature of caries it will be interesting to see the *in vivo* efficacy of this targeted approach.

Few clinical studies using HDPs to treat oral diseases have, as yet, been published. IB-367, which was based upon the porcine cathelicidin, protegrin, demonstrated safety and efficacy in Phase I and Phase II clinical trials for treatment of oral mucositis, although it failed in Phase III trials (Falla and Zhang, 2004; Mosca *et al*., 2000). A topical gel of the histatin 5 derivative, P-113, reduced experimental gingivitis in human subjects (Paquette *et al*., 2002) and a P-113 mouthrinse for the treatment of gingivitis has undergone successful clinical trials (Mickels *et al*., 2001).

Plaque bacteria reside in biofilm communities and some *in vitro* studies have demonstrated peptide activity against biofilm-grown organisms or inhibition of biofilm formation (Altman *et al*., 2006; Eckert *et al*., 2006; Hao *et al*., 2005; Porat *et al*., 2006; Wei *et al*., 2006). Oral bacteria additionally form biofilms of clinical significance on implants and dentures within the mouth and HDPs are being used to develop novel materials with reduced propensity for biofilm formation. Attachment of histatin 5 to denture base material inhibited formation of biofilms by *C. albicans* (Yoshinari *et al*., 2006) and histatins, mucin-derived peptides, and defensins have been incorporated into multilayer polyelectrolyte films with the aim of developing biocompatible antimicrobial coatings for implants (Etienne *et al*., 2004; Lindh *et al*., 2007).

The need for new therapeutic agents to combat microbial disease against a background of increasing resistance to conventional antibiotics has also resulted in approaches being pursued that do not rely on direct antimicrobial activity and these are well demonstrated by new approaches to treatment of periodontal diseases. There has been a general paradigm shift away from an emphasis on the acquisition and activities of specific periodontal pathogens towards an ecological- and microbial community-based approach to understanding periodontal diseases, which also acknowledges the significant roles of host responses and commensal populations. This has important implications for approaches to therapy and has enhanced the possibilities of developing novel strategies through manipulation of normal microbiota and modulation of host immune responses. Thus, new therapeutic approaches have been developed using antiproteinases, anti-inflammatory drugs and bone-sparing or antiresorptive drugs (bisphosphonates), novel agents that inhibit the signaling pathways central to the exaggerated inflammatory response in periodontitis (Kantarci *et al*., 2006; Kirkwood *et al*., 2007; Van Dyke and Serhan, 2003) and analogues of lipoxin and resolvins (derived from omega-3 fatty acid precursors) with anti-inflammatory and immunomodulatory activities (Van Dyke and Serhan, 2003). Recently, small HDP-based peptides that function solely through immune modulation (Scott *et al*., 2007) or through their ability to bind to and suppress responses to LPS have been developed for use as anti-infectives (Falla and Zhang, 2004; Hancock and Sahl, 2006). The anti-LPS and immunosuppressive properties of HDP derivatives should find applications in the treatment of periodontal and other oral inflammatory conditions. In addition, Brogden *et al*. (2003) are exploring the use of intranasal administration of defensins with relevant antigens to promote a mucosal adaptive immune response effective in treating or preventing periodontal disease.

VII. CONCLUSIONS

HDPs are important in defense of tissues throughout the human mouth. Like resident microbial populations, HDPs are diverse, species-specific, and site-specific (Devine and Hancock, 2002), and they have evolved in response to selection pressures exerted by resident and pathogenic microbial populations (Hughes, 1999). Their site-specific expression is likely to be a significant contributor to the tissue tropism displayed by microorganisms throughout the body. Currently there is an impetus to understand host–microbe interactions in health as well as disease, and the mechanisms underlying the ability of host tissues to tolerate resident microbial populations while maintaining efficient responses to pathogens are of fundamental importance. Oral populations are the best characterized

biofilms of relevance to human health and disease and major advances have been made in characterizing populations that are specific to certain oral sites and to defined stages of disease. These developments should significantly enhance our ability to understand host–microbe interactions and help clarify the roles of HDPs in determining or regulating host–microbe relationships.

No consistent correlation has emerged so far between oral commensalism and susceptibility to, or induction of HDPs. Equally, pathogenic bacteria exhibit a variety of strategies for evading or surviving HDPs. However, only a small number of oral commensal species and strains have been studied. Also, to truly understand the *in vivo* roles of HDPs in health and protection against infection we need to understand their interactions with organisms in complex biofilm communities, not with planktonic monocultures as has predominantly been the case to date.

Elucidating the roles of HDPs in protection from oral diseases is challenging because of the complexity and polymicrobial nature of many of these diseases. Nonetheless, some associations have begun to emerge, for example between high levels of expression of salivary histatins and HNPs and low caries experience, as well as between high epithelial expression of hBD 2 and periodontal health. However, it is clear that we need to better understand the factors that contribute to the high levels of intersubject and interstrain variability observed. Many studies of periodontitis have generated conflicting results. This is a complex chronic disease that displays periods of activity and stability. Studies of the early stages of disease and of sites coming out of remission into a period of inflammatory activity will be important to clarify the roles of HDP expression in periodontal health and disease.

The antimicrobial and antibiofilm activities of HDPs, as well as their immunomodulatory properties, are being exploited to develop novel therapeutic agents and biomaterials to combat infections. For such developments, natural human peptides such as hBDs, LL-37, and histatins are thought by some to be advantageous because they are likely to be biocompatible. However, their inhibition by monovalent and divalent cations, mucins, saliva, and serum (Hancock and Sahl, 2006; Maisetta *et al*., 2005) must be overcome, as must their sensitivity to host and microbial proteases. Judicious design of novel peptide analogues is overcoming these problems, as well as those associated with cost of production. Although resistance to HDPs is rare and does not emerge rapidly, it is possible that long- or short-term resistance may develop with prolonged exposure to increased concentrations of HDPs (Perron *et al*., 2006; Shelburne *et al*., 2005). The occurrence of such phenomena *in vivo* and the risk that even short-term resistance poses must be assessed before human innate defense molecules are used in treatment of microbial disease.

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